Autosomal recessive retinal dystrophies:
genotypes & phenotypes

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Autosomal recessive retinal dystrophies: genotypes and phenotypes
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Contents

Abbreviations

A2E	*N*-retinylidene-*N*-retinylethanolamine
aa	amino acid
AAV	adeno-associated virus
ABCA4	ATP-binding cassette transporter, subfamily A, member 4 (retina specific)
ABCR	ATP-binding cassette transporter (retina-specific)
adRP	autosomal dominant retinitis pigmentosa
AGRA	Affymetrix's GeneChip CustomSeq Resequencing Array
AIPL1	arylhydrocarbon-interacting receptor protein-like 1
AMD	age-related macular degeneration
APEX	allele-specific primer extension
ar	autosomal recessive
arCRD	autosomal recessive cone-rod dystrophy
arRP	autosomal recessive retinitis pigmentosa
ATP	adenosine triphosphate
BE	both eyes
bp	base pair
cd/m^2	candela per square metre
cDNA	complementary deoxyribonucleic acid
CF	counting fingers
cGMP	cyclic guanosine-monophosphate
cM	centiMorgan
CRB1	Crumbs homologue 1
CRD	cone-rod dystrophy
CRX	cone-rod otx-like photoreceptor homebox transcripiton factor
dHPLC	denaturating high-performance liquid chromatography
DNA	deoxyribonucleic acid
ERG	electroretinogram
FAG	fluorescein angiogram
FR	fovea reflex
GUCY2D	retinal-specific guanylate cyclase 2D
HM	hand movements
Hz	Hertz
IS	inner segment
ISCEV	International Society for Clinical Electrophysiology of Vision
kb	kilobases
LCA	Leber congenital amaurosis
LE	left eye
LP	light perception
LRAT	lecithin retinol acyltransferase
Mb	megabases
MERTK	mer tyrosine kinase protooncogene
mfERG	multi-focal electroretinogram

mM	millimolar
MR	macula reflex
mRNA	messenger ribonucleic acid
mu	mutant
NLP	no light perception
NR	non recordable
nt	nucleotide
ONL	outer nuclear layer
OS	outer segment
PCR	polymerase chain reaction
Ph	photopic
PPRPE	preserved para-arteriolar retinal pigment epithelium
PR	photopic responses
RDH12	retinol dehydrogenase 12
RE	right eye
RFLP	restriction fragment length polymorphism
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
RPE65	retinal pigment epithelium-specific protein, 65kD
RPGRIP1	retinitis pigmentosa GTPase regulator-interacting protein
Sc	scotopic
SE	spherical equivalent
sec	second
SNP	single nucleotide polymorphism
SR	scotopic responses
SSCP	single strand conformation polymorphism
STGD1	autosomal recessive Stargardt disease type 1
TULP1	tubby-like protein 1
VA	visual acuity
VF	visual field
wt	wild type
xlRP	X-linked retinitis pigmentosa
yr	year

1 General introduction

Introduction

Inherited retinal dystrophies afflict approximately one in every 2000 individuals[1] and show wide clinical and genetic heterogeneity. Until recently, classification of these diseases was only based on their clinical presentation and inheritance pattern. After the identification of the first retinal disease genes in the early nineties[2,3] a refined classification based upon the underlying genotype seemed rational, but this attempt is still in its infancy. Up to now more than 100 genes responsible for human retinal diseases have been discovered and many more await discovery. Paradoxically, this has raised as many questions as it has answered, for mutations in different genes may result in similar phenotypes, while on the other hand mutations in one gene can lead to different diseases, even with a different mode of inheritance.

Detailed knowledge of the molecular genetic causes of inherited blindness is essential for understanding the underlying cellular and molecular mechanisms involved in these types of retinal dystrophies, giving more insight into both normal and pathologic RPE and photoreceptor function and development. Knowledge of final common pathways may lead to the discovery of new genes involved in hereditary retinal dystrophies, and eventually all this information will pave the way for the development of therapeutic interventions. Improved understanding will also provide an increasingly accurate basis for patient counseling.

In this thesis patients with various types of retinal dystrophies have been studied clinically and at the molecular level, in order to improve genotype-phenotype correlations.

1.2 Anatomy and physiology of the retina

The complex process of vision has intrigued mankind for centuries and the first records date back to Aristotle (4th century BC). A first realistic understanding of the function of the eye began after the gross anatomy of the eye had been established. We have come to realize that vision is possible through very complex, finely tuned sets of molecular cascades and cellular machineries.

In the macro-anatomy of the posterior part of the globe, three major cell-layers are recognizable. The outer layer, the sclera, is mainly formed by collagen fibrils providing the globes firmness. The middle layer, the choroid, is composed of arterioles and venules and a fenestrated capillary network that nourishes the outer part of the third and most inner layer, the retina (figure 1.1). The retina is the most vital layer for vision since it contains neurons that are sensitive to light and are capable of amplifying, integrating, and transmitting visual signals. The retina is a highly organized structure that comprises two distinct layers: an outer layer, the retinal pigment epithelium (RPE) and the adjacent inner layer, the neurosensory retina.

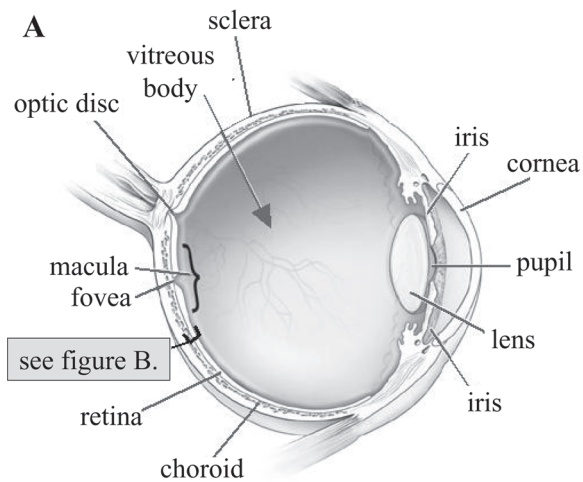

Figure 1.1a Anatomy of the eye

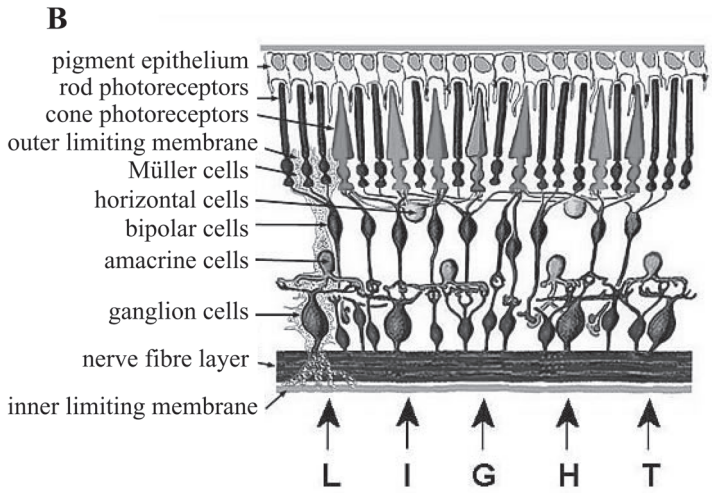

Figure 1.1b Structure of the retina
http:// webvision.med.utah.edu

1.2.1 The retinal pigment epithelium

This outer pigmented layer is situated between the choroid and the photoreceptor cells of the neurosensory retina (figure 1.1b). The RPE is a highly polarized and specialized epithelium that serves as a multifunctional and indispensable component of the eye. This single cell layer is formed by versatile hexagonally shaped cuboidal cells and originates as an anterior neural plate derivative from the neuro-ectoderm[4,5]. This makes RPE cells the only pigmented cells of the body that are not derived from the neural crest[6]. RPE cells are characterized by the presence of lysosome-related organelles that are committed to the biosynthesis and storage of melanin pigments, the melanosomes[5]. Melanin is a crucial component in the absorption of stray light that enters the eye.

The apical side of the RPE cell has multiple villous processes into which the outer segments of cones and rods are embedded. The narrow space between the RPE and the photoreceptor cells is filled with a hyaluronan-rich extracellular matrix formed by apical secretion of hyaluronan by RPE cells[7]. This interphotoreceptor matrix forms a structural link between the neural retina and the RPE[8].

The RPE monolayer is responsible for many functions such as: 1. The participation in the outer retinal blood-ocular barrier, 2. The uptake, processing, transport and release of vitamin A (retinol), 3. Maintaining the adhesion and water and ions flow between the neural retina and the choroid, 4. Protection against free radicals, 5. Maintenance of the subretinal space, and 6. Phagocytosis of the outer segment discs of the photoreceptors and ensuring their renewal. Based on these functions,

the RPE plays a key role in the maintenance of the overlying photoreceptor layer from the neurosensory retina[9,10].

1.2.2 The neurosensory retina

The complex neurosensory retina comprises six types of neurons: the amacrine cells, bipolar cells, ganglion cells, horizontal cells, Müller cells and the photoreceptor cells. These cells together form three distinct layers of neurons (nuclear layers) and two different layers of synapses (plexiform layers). The outer nuclear layer contains cell bodies of the photoreceptors, the inner nuclear layer contains cell bodies of the amacrine, bipolar, horizontal and Müller cells (the interneurons) and the ganglion cell layer is formed by cell bodies of the ganglion cells. These nerve cell layers are divided by the layers of synapses: the outer plexiform and the inner plexiform layer.

The neurosensory layer is responsible for the detection and transmission of photoreceptor-generated signals to the central visual pathway. Light-evoked signals are transferred from the synaptic terminals of the photoreceptor cells to the bipolar and the horizontal cells. The horizontal cells provide lateral interactions in the outer plexiform layer. The bipolar cells transfer the light signals to the inner plexiform layer into the dendrites of amacrine and ganglion cells. Müller cells span the entire neurosensory retina. These are glial cells involved in several essential activities for normal retinal function. Ganglion cell dendrites collect the signals of the bipolar and the amacrine cells and their axons finally transmit a nerve impulse to the central visual pathway[11].

1.2.3 The photoreceptor cell

Mammals have two main types of highly specialized neuro-epithelial photoreceptor cells, the rods and cones. Photoreceptor cells are responsible for transducing light (electromagnetic signal) into a molecular signal, and then into a neurological (electrical) signal. The human retina contains approximately 120 million rods and 6 million cones. Whereas rods mediate dim-light vision and sense contrast, brightness and motion, cones function in bright light and subserve fine and spatial resolution, and realize the differentiation between spectral modulations (color vision).

The density of rods is much greater than the density of cones throughout most of the retina, with the exception of the fovea. This is a highly specialized region of the central retina that measures about 1.2 millimeters in diameter with the highest receptor packing density, and exclusively contains cones. This partitioning is in concordance with the distinct function of rods and cones. In the fovea the high spatial resolution (high visual acuity) is ensured by a nearly one-to-one relationship between a cone photoreceptor cell, its bipolar and ganglion cell and its nerve fiber. The peripheral photoreceptors are not individually coupled to a bipolar cell or to a

single ganglion cell but are grouped and coupled. This so-called convergent wiring is necessary in order to maintain detection levels of small amounts of light.

Both rods and cones contain an outer (OS) and an inner segment (IS), a nucleus and a synaptic terminal, but are histological distinguishable by the shape and position of the nucleus. Photoreceptors are characterized by the presence of discs in their outer segments, which arise as a series of invaginations of the cell's plasma membrane. Each outer segment contains roughly 1000 discs. Approximately 10% of these are renewed on a daily basis. They are formed at the IS/OS junction and released to the RPE at the apical end which then binds, ingests and digests the shedded discs. This makes the RPE cell perhaps the most efficient phagocyte of the human body[4].

Crucial molecules and proteins involved in the activation and quenching of the visual cascade (the phototransduction cascade), are embedded in these photoreceptor discs. One such indispensable component are visual pigment molecules which are necessary for capturing light and the convertion of light into an electrical signal (figure 1.2). Rods and cones harbor different types of pigment. The rods contain the rhodopsin protein whereas cones contain one of three different types of iodopsin (red, green and blue), which respond selectively to photons in different regions of the visible light spectrum, enabling color vision.

The inner segments of the photoreceptors contain the metabolic machinery, like mitochondria, ribosomes and the Golgi complex. The cellular compartments of the light sensitive outer segments are linked to the biosynthetically active inner segments by a thin cylindrical cytoplasm bridge containing an immotile cilium. This connecting cilium serves as a barrier for membrane components and soluble proteins and thus regulates and facilitates the free diffusion of OS proteins synthesized in the IS. Furthermore, since this is the only intracellular bridge between the two segments, intracellular exchanges between the segments are forced to occur through the connecting cilium.

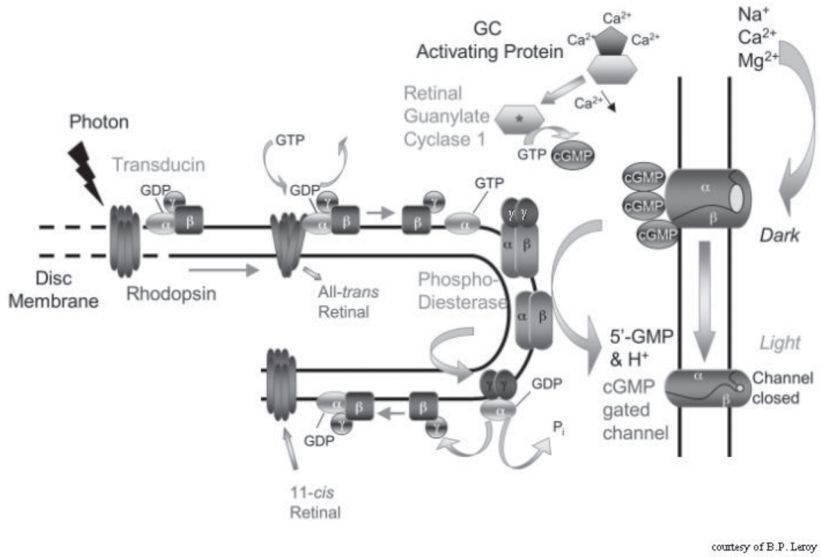

Figure 1.2 The phototransduction cascade
After the light activation of rhodopsin consecutive reactions occur. When cGMP is hydrolized the permeability of the plasma membrane cGMP channels decreases. This leads to hyperpolarization of the photorecptor cell membrane and decreased neurotransmitter release at synapses.

1.2.4 The phototransduction cascade

The phototransduction system consists of a cascade of successive molecules involved in the processes of light activation and inactivation. Activation occurs when light is absorbed by visual pigments to which a highly photosensitive vitamin A analogue is covalently bound (11-*cis*-retinal). Vitamin A is converted to 11-*cis*-retinal in the RPE and is recycled through the visual cycle. Light absorption results in the isomerization of 11-*cis*-retinal to all-*trans*-retinal, which subsequently forms a photoactive visual pigment (metarhodopsinII), which decays through several intermediates[12]. One of these intermediates activates transducin, a G-protein specific to photoreceptor cells which in its turn activates a cGMP phosphodiesterase resulting in the hydrolysis of the “second messenger” cGMP to 5’-GMP[13]. For the biosynthesis of rod cGMP phosphodiesterase, the chaperone aryl hydrocarbon receptor-interacting protein-like 1 protein (AIPL1) is required[14]. The hydrolysis of cGMP after photon stimulation leads to a decrease of cGMP reducing the permeability of the photoreceptor plasma membrane cGMP-gated cation channels. This initiates a significant lowering of the intracellular calcium levels and as a consequence the photoreceptor cell plasma membrane is hyperpolarized. This then leads to a decreased release of the neurotransmitter glutamate at the synaptic terminal (figure 1.2).

The inactivation of the light-induced response, the recovery phase, occurs via several mechanisms leading to the dissociation of all-*trans*-retinal from the visual pigments, the phosphorylation of rhodopsin by rhodopsin kinase and the subsequent binding of rhodopsin to arrestin and finally to the restoration of the cytoplasmic levels of cGMP by guanylyl cyclase in order to reset the light sensitivity of photoreceptor cells[15,16] (figure 1.2). One of the key enzymes in this process is probably RDH12[17,18].

1.2.5 The visual (retinoid) cycle

Vitamin A (all-*trans*-retinol) is a crucial component for the phototransduction cascade and is absorbed via dietary intake and converted to 11-*cis*-retinal in the RPE. The metabolism of vitamin A and the cycling of the retinoid analogues between the RPE and the photoreceptor cells is a complex process called the visual cycle. The visual cycle begins following a light response, which leads to the release of all-*trans*-retinal from the activated visual pigment. In the lumen of the photoreceptor cell disc membranes, all-*trans*-retinal reacts with phosphatidylethanolamine and forms N-retinylidene-phosphatidylethanolamine (A2N)[19]. Through a flippase activity of the ABCR protein, A2N is translocated from the inner to the outer half of the lipid layer of the photoreceptor discs and all-*trans*-retinal is released to the cytoplasm[20,21] (figure 1.3). In the cytoplasmic space, all-*trans*-retinal is reduced to all-*trans*-retinol by an all-*trans*-retinol dehydrogenase (RDH) and is transported to the RPE[22,23]. Lecithin retinol acyltransferase (LRAT) is the first enzyme in the RPE portion of the retinoid cycle and esterifies a phosphatidylcholine to all-*trans*-retinol in order to form all-*trans*-retinyl esters[24,25]. These esters are then isomerohydrolysed to from 11-*cis*-retinol[26]. A crucial enzyme in the isomerohydrolyses of all-*trans*-retinyl esters is the retinal pigment-specific 65kDa protein (RPE65)[27]. Finally, 11-*cis*-retinol dehydrogenase (11-*cis*-RDH) converts 11-*cis*-retinol in 11-*cis*-retinal for which the presence of the cellular retinaldehyde-binding protein (CRALBP) is necessary[28]. 11-*cis*-retinal eventually exits the RPE, traverses the subretinal space and then re-enters the photoreceptor outer segment where it is recombined with an opsin protein to yield a light-sensitive rhodopsin molecule (figure 1.4).

Sustained vision in bright illumination requires continuous regeneration of the visual pigments in cones. It has been suggested that the maximum throughput of this visual cycle is too slow to keep up with the high demand and a novel visual cycle for sustained daylight vision was proposed after studying cone-dominant squirrel and chicken retina's. This pathway is located outside of the RPE in cone outer segments and Müller cells, and involves three catalytic activities in cone membrane fractions, regenerating opsin photopigments at a 20-fold faster rate[20].

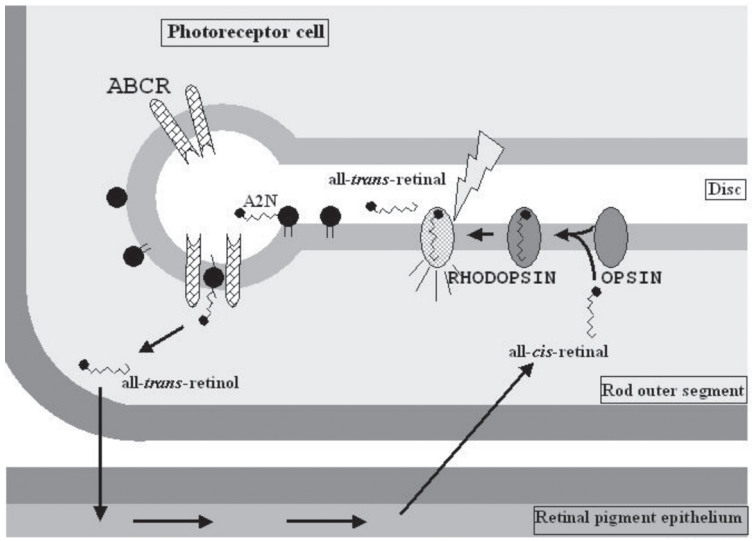

Figure 1.3 The function of the ABCR protein
After light activation all-trans-retinal is released from the activated visual pigment, which will subsequently lead to formation of N-retinylidene-phosphatidylethanolamine (A2N). Through the activity of ABCR all-*trans*-retinal is translocated from the intra disc lumen to the cytoplasm. In the cytoplasmic space, all-*trans*-retinal is reduced to all-*trans*-retinol and is transported to the RPE and finally returned to the photoreceptor outer segment where it is combined with an opsin molecule.

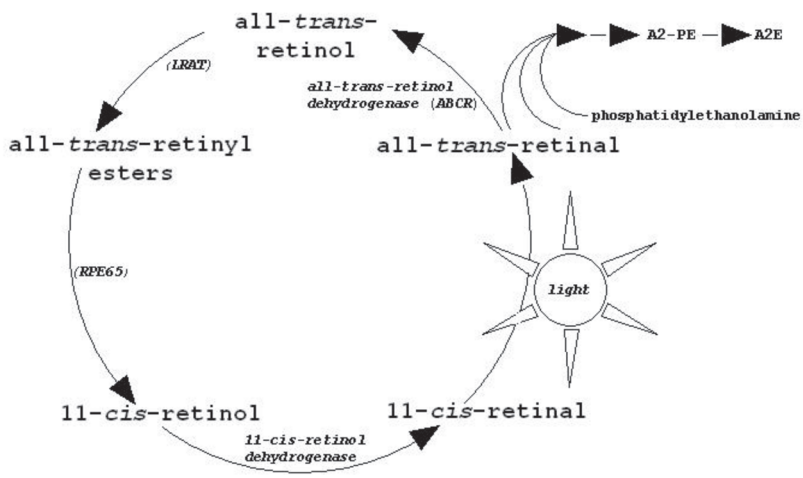

Figure 1.4 Schematic of the (re)cycling of vitamin A (all-*trans*-retinol)
A2E: N-retinylidene-phosphatidylehanolamine; A2-PE: phosphatidylethanolamine-bisretinoid; ABCR: ATP-binding cassette transporter; *LRAT*: lecithinretinol acyltransferase; *RPE*65: RPE-specific 65kDa protein.

1.3 Phenotypical assessment

Inherited retinal dystrophies display a wide clinical spectrum. Their diagnosis is usually based on the age of onset, the type and severity of symptoms, their progression, the family history and a thorough ophthalmologic exam, in particular the retinal appearance. In addition to the clinical aspects of the phenotyping effort, functional retinal assessments such as visual acuity, electroretinography, visual field measurements, and color vision testing, are of great importance to identify the nature of the disorder and for patient's follow-up. Nevertheless, it is often difficult to establish a clear-cut clinical diagnosis as several retinal dystrophies have significant clinical overlap.

Furthermore, although the primary defects may be very different at the genetic and molecular level, there is evidence that only a few final common cell death pathways exist so that there is convergence from the initial insult to the retinal cells to the final cell death pathways. These pathways are highly controlled and complex systems of effector cascades that converge to a common death execution program of single cells (apoptosis). The induction of apoptosis is very complicated and as yet poorly understood. It has been proposed to arise within the diseased cell or to be triggered by extracellular signals. Both of these conditions may be enhanced by exogenous factors such as light exposure, nutrition or toxic agents[29,30].

1.3.1 Functional testing

Visual acuity (VA) is measured under standardized conditions and provides important information on the spatial resolving capacity of the central visual system. In general, VA is measured by asking the person to discriminate letters (or figures) of known visual angle at standard distances under standard illumination. Snellen charts are the universal standard, which uses a pragmatic sequence of letter sizes. The small steps represent the range of normal vision and the larger steps the range for poorer vision. Letter spacing varies and accordingly the number of letters per line. ETDRS (Early Treatment Diabetic Retinopathy Study) charts uses proportional spacing (geometric or logarithmic progression), meaning that step sizes are constant at all levels of vision, so that the relative crowding and contour interaction remains the same for all lines and only the magnification varies with a logarithmic step size. The outcome is presented in a quotient, d/D, in which d represents the distance of the patients eye to see the letter and D represents the distance (meter) from which a "normal" eye would see the specific letter. Preverbal children cannot perform this type of testing and many age-adapted tests are now available. One such test uses the Teller visual acuity cards, which measures preferential looking at lines of which the VA is determined by the frequency of the line spacing. Central, steady and maintained fixation on a target or a child's fixing and following behaviour are used as indicators of vision in newborns.

Another test of great importance in the field of retinal dystrophies is the full-field electroretinogram (ERG), which measures a mass electrical response of an action potential produced by the whole retina when stimulated by illumination. The ERG can be elicited in both light-adapted (photopic) and dark-adapted (scotopic) states. Photopic recordings are the exclusive responses of the cones. Under scotopic conditions, isolated rod responses can be measured as well as mixed cone-rod responses. A normal ERG result is a biphasic waveform consisting of an a-wave and b-wave. The a-wave originates in the photoreceptors and represents a negative deflection from baseline and identifies the closing of the cGMP-gated channels, whereas the b-wave is a positive deflection from baseline and originates in the post-receptor cell layer including the bipolar cells and Müller cells. ERGs made for the purpose of studies presented in this thesis were made under conditions dictated by the "International Society for Clinical Electrophysiology of Vision" (www.iscev.org). Multifocal ERG (mfERG) allows simultaneous recordings of 64 local cone ERGs and in contrast to the conventional ERG, specific information on multiple small areas is gathered.

Goldmann kinetic perimetry measures suprathreshold retinal sensitivity and gives a definition of the spatial retinal localization of the retinal disorder.

Color vision tests discriminate between separate types of cone defects and indicate protan (red), deutan (green) and tritan (blue) cone photoreceptor abnormalities.

1.4 Clinical classification of selected retinal dystrophies

A straightforward classification of inherited retinal dystrophies is complicated since the different disorders comprise a spectrum and show overlapping characteristics. The general clinical features of Leber congenital amaurosis, retinitis pigmentosa, cone-rod dystrophy, and Stargardt disease will be discussed since they are the subject of this thesis. Some of these diseases may be a component of multisystem disorders (syndromic disease) but for the purpose of this thesis only the non-syndromic forms will be discussed.

1.4.1 Leber congenital amaurosis

Leber congenital amaurosis (LCA) was first described by Theodor Leber in 1869[31]. He described patients who were congenitally blind and who did not have obvious fundus abnormalities on initial examination. Later in life many of these children developed retinitis pigmentosa-like fundus changes. LCA is the earliest and most severe form of all inherited retinal dystrophies leading to blindness or severe visual impairment from birth with an incidence of 3:100.000[31]. It is postulated that LCA accounts for

more than 5% of all inherited retinal dystrophies and affects approximately 20% of the children attending schools for the blind around the world[32].

LCA is characterized by poor fixation in the first 6 months of life, a sensory (wandering) nystagmus, amaurotic pupils and a variable fundus appearance. The fundus changes may vary from completely normal (even later in life), to mild pigment mottling with mild vascular attenuation, to a full blown RP type fundus. An absolute diagnostic prerequisite is a severely reduced or non-detectable ERG in the first year of life. LCA may be accompanied by other ocular features such as eye-poking (the oculodigital sign of Franceschetti), ptosis, strabismus, high hyperopia, high myopia, microphthalmos, cataracts early in life, keratoconus, macular coloboma, and disc edema[33].

Most clinical studies show that LCA patients have relatively stable visual function, with some patients deteriorating (especially those with macular colobomas, cataracts, keratoconus, etc), and some patients improving. The improvement usually does not last and the prognosis is poor.

Histological studies appear to corroborate the clinical studies and have shown LCA to be a heterogeneous group of disorders, which may be classified in three functional subtypes according to the underlying pathological processes; aplasia (or agenesis) due to an abnormal embryological formation of the photoreceptors; a degeneration caused by an early progressive photoreceptor cell death, and third, a dysfunction in which the retinal anatomy remains normal but a key biochemical messenger is missing. This is of significance in the development of future therapies.

1.4.2 Retinitis pigmentosa

The name retinitis pigmentosa (RP) was coined by the Dutch physiologist and ophthalmologist Donders in 1857[34], but is actually a misnomer since the retinal dystrophy is not caused by an inflammation. RP patients form the largest group of patients with inherited retinal disorders worldwide with a prevalence of 1:3000[35]. RP comprises a surprisingly heterogeneous group of in general progressive disorders that primarily affect the function of the photoreceptors and the RPE, with a large variability in age of onset, progression, retinal appearances and eventual visual outcome. Patients usually present with nyctalopia (night blindness), followed by or concurrent with peripheral visual field (VF) loss. The long-term prognosis is generally poor for most RP patients since maculopathy will eventually affect the central visual acuity. Some patients lose light perception at the end stage of the disease.

The ophthalmoscopic features typically consist of bone spicule formation in the retina which represents pigment granules released from RPE cells that have migrated to perivascular sites in the inner retina secondary to photoreceptor death[36]. Hyperpigmentation of the areas of the retina may alternate with areas

of hypopigmentation or intraretinal white dots. The posterior pole may also be relatively spared. The attenuation of the retinal arterioles and veins is probably the result secondary to photoreceptor death. Finally, optic nerve alterations described as waxy pallor appearance or optic atrophy are often seen. The loss of pigment of the RPE and the loss of choriocapillaris enables visualization of the larger choroidal vessels, which may appear whitish (choroidal sclerosis). In addition to these classical symptoms, RP may be associated with early posterior subcapsular cataracts, early vitreous degeneration, hyaline bodies or drusen of the optic disc, and cystoid macular edema[37].

Crucial in the diagnosis of RP are results captured by the electroretinogram (ERG) showing abnormal rod responses. Later in the disease course both cone- and rod-mediated responses are reduced and eventually may become undetectable.

Visual field defects are variable and may be classified into several types. Grover and co-workers[38] classified visual fields of 86 RP patients into three patterns. Pattern I shows a characteristic progressive concentric loss, pattern II shows an initial superior loss subsequently leading to an arcuate scotoma winding around the central area from either the nasal or temporal side. The main motif of pattern III progression is characterized by an incomplete or complete ring scotoma in the midperipheral areas. In their study the pattern of visual field loss is consistent within members of the same family. A specific pattern of photoreceptor cell death may be explained by the underlying molecular mechanism initiating cell apoptosis[38].

1.4.3 Cone-rod dystrophy

In contrast to the clinical presentation of RP, patients with cone-rod dystrophy (CRD) initially have a predominant loss of cone function. Their symptoms reflect cone dysfunction and consequently patients usually present with photoaversion and dyschromatopsia (in adolescence or early adult life), day blindness, and progressive loss of visual acuity. The degeneration of rod photoreceptors is by definition later in the disease course and accompanying symptoms like night blindness may then occur.

The retinal findings of CRD patients may be surprisingly subtle, especially early in the disease process. Funduscopic examination may show either minimal or non-specific pigmentary changes within the fovea, an atrophic-appearing macular lesion (which may have a bull's-eye-like appearance), or even more diffuse pigmentary degenerative changes involving both the macula and more peripheral regions of the retina[39]. This may be accompanied by mild attenuation of the retinal vessels. Perimetry shows central scotomas and eventually large paracentral scotomas and mild peripheral constriction.

ERG is crucial in the diagnosis of CRD, which demonstrates a predominant reduction of the cone rather than the rod mediated response. It may be difficult

to clinically distinguish between advanced CRD and advanced RP since their funduscopic appearances may be similar and both rod and cone ERGs may be non-recordable. The long-term prognosis of CRD is often poor.

1.4.4 Stargardt disease

Stargardt disease (STGD1) was first described by Karl Stargardt in 1909[40] and is the most common hereditary juvenile macular dystrophy with a prevalence of approximately 1:10.000[41]. The disease is usually characterized by a bilateral loss of visual acuity in early childhood or early adolescence.

The initial fundoscopy may reveal no obvious abnormalities whereas in later stages of the disease the typical subretinal yellowish flecks, which are variable in size and shape (and have a transient character), may be present in the posterior pole and may extend as far as the equator. As the disease develops, RPE atrophy in the macular region may show up as a beaten bronze appearance or a bull's eye pattern. Pigmentary changes in the midperiphery may also be present.

Electrophysiologic dysfunction is not uncommon in STGD1 patients even in presence of normal visual acuity. Fluorescein angiography (FAG) may also be useful, since the typical yellow-white flecks either appear as hypofluorescent or demonstrate an irregular pattern of fluorescence. A more important and typical FAG feature in STGD1 is the obstruction of the normal choroidal background fluorescence formed by the accumulation of lipofuscin that is present in 50-85% of the patients (choroidal silence).

1.5 Molecular genetics

In 1953 research conducted by J. Watson, F. Crick, M. Wilkin and R. Franklin led to the important discovery of the double helix structure of DNA[42-44]. DNA is a linear polymer with two complementary strands, each made up of a linear array of the purine bases guanine (G) and adenine (A) and of the pyrimidine bases cytosine (C) and thymine (T). The specific genetic information is captured in the unique sequence of these four bases. A gene consists of several hundreds to thousands of basepairs. DNA is transcribed into mRNA, which in turn encodes a protein. A change (mutation) in one or a few of the bases of a gene can result in a change of the structure or stability of the mRNA or in a change of the encoded protein, and this may cause disease. Mutations derived from the parental DNA can be passed on to the next generation and subsequently cause inherited diseases.

DNA carries genetic information and, together with nuclear proteins, is packaged into chromosomes. In humans the cell nuclei contain 23 pairs of chromosomes, 22

pairs of autosomes and one pair of sex-chromosomes (XY in males, XX in females). The chromosomes are distinguishable because they carry nucleotide variations in ~ 1 in 1000 base pairs. Each member of a pair is derived from the father or mother.

The human genome actually comprises two types of genomes; the above-described nuclear genome carries approximately 30,000 genes (total size is 3,200,000 kb) and a much smaller mitochondrial genome with 37 genes (total size is 16.6 kb). Mitochondria reside in the cytoplasm and are only transmitted through females.

1.5.1 Inheritance patterns

The different modes of monogenic (single-gene) disorder inheritance are described in this paragraph. In autosomal dominant (ad) inherited disease, a mutation in one of the two gene copies leads to disease. A carrier of the mutation, in most cases, will develop the disease and has a 50% probability of passing the mutation on to the next generation.

When both autosomal genes are required to harbor a disease-associated mutation the mode of inheritance is autosomal recessive (ar). A heterozygous carrier of a mutated and a normal gene will not develop the disease. A carrier couple has a 25% probability for any of their children to develop the disorder. A person suffering from the disease will pass one mutation on to the next generation, which automatically renders children carriers of the mutation.

Men and women differ in their twenty-third pair of chromosomes. Women carry two fully homologous X chromosomes; men carry the X and Y chromosomes, which are fully homologous at the tips of the short arms of the X and Y chromosomes (~3 Mbp; pseudo-autosomal region) and only partially homologous in other regions. In females most genes on one X chromosome are silenced as a result of X-inactivation. Apparently, the product of one of two gene copies is sufficient and necessary for normal function. The process of X-inactivation in female cells is initiated during early development and is controlled by the X-inactivation centre. The Y chromosome harbors the critical male-determining gene, sex-reversed Y (*SRY*). Since males are hemizygous for almost all genes on the X-chromosome, except for those located on the pseudo-autosomal region at Xpter, a mutation in a gene on the X chromosome in males, in most cases will lead to disease (X-linked). Female offspring of an affected male are obligate carriers but in most cases unaffected. Male offspring are unaffected since they inherit the Y-chromosome. The mitochondrial DNA is exclusively maternally inherited.

All three types of Mendelian inheritance are possible in RP. The most frequently occuring mode of inheritance is autosomal dominant, followed by autosomal recessive and X-linked RP. The majority of patients however are so called isolated cases without any family history of RP. In these patients either of the above

mentioned modes of inheritance is possible. Only once a family with digenic inherited RP has been reported[45]. The affected family members had mutations in two unlinked photoreceptor-specific genes (*ROM1* and *peripherin/RDS*), and were double heterozygous[45].

In progressive cone-rod dystrophy the mode of inheritance is similar to RP with again predominance for isolated cases followed by autosomal dominant, some autosomal recessive and some X-linked cases. LCA is mostly inherited in an autosomal recessive manner, although a few cases with autosomal dominant inheritance are described. STGD1 is an autosomal recessive inherited disease. Mitochondrial inheritance is not shown in the above-mentioned isolated diseases.

1.5.2 Genetic heterogeneity

Since the studies described in this thesis concentrate on autosomal recessively inherited retinal dystrophies, only the genetics of arRP, arCRD, LCA, and STGD1 will be discussed. ArRP, arCRD, and LCA display wide genetic heterogeneity. The first arRP gene discovered was the rhodopsin gene[46] and now, many years after this discovery, 19 arRP genes have been cloned, 5 are mapped and many more await discovery.

The identification of the first LCA-causing gene in 1996[47] was followed by the identification of an additional seven genes. Together they account for approximately 45%[48] of the cases suffering from LCA, implicating that 55% of the LCA cases are caused by genes that are yet to be discovered.

In arCRD the *ABCA4* gene has been implicated in 40-50% of the cases, but many other genes are expected to be involved. STGD1 is the only disease studied here that is considered to be caused solely by mutations in the *ABCA4* gene[49]. The causative genes and loci of arRP, arCRD, LCA and STGD1 are listed in table 1.1.

Table 1.1 The causative genes and loci for arRP, arCRD, LCA and STGD1
* Mutations are associated with juvenile RP
For an overview, see: www.ncbi.nlm.nih.gov/Omim/ and www.sph.uth.tmc.edu/Retnet/

ar retinitis pigmentosa		
genes	*ABCA4*	1p21-p13
	CERKL	2q31.2-q32.3
	CNGA1	4p12-cen
	CNGB1	16q13
	CRB1	1q31-q32.1
	LRAT	4q31
	MERTK	2q14.1
	NR2E3	15q23
	NRL	14q11.1-q11.2
	PDE6A	5q31.2-q34
	PDE6B	4p16.3
	RGR	10q23
	RHO	3q21-q24
	RLBP1	15q26
	RP1	8q11-q13
	RPE65	1p31
	SAG	2q37.1
	TULP1	6p21.3
	USH2A	1q41
loci	RP22	16p12.3-p12.1
	RP25	6q14-q21
	RP28	2p15-p11
	RP29	4q32-q34
	RP32	1p34.3-p13.3

Leber congenital amaurosis		
genes	*AIPL1*	17p13.1
	CRB1	1q31-q32.1
	CRX	19q13.3
	GUCY2D	17p13.1
	IMPDH1	7q32.1
	RDH12	14q23.3
	RPE65	1p31
	RPGRIP1	14q11
loci	LCA3	14q23.3
	LCA5	6q11-q16
	LCA9	1p36
genes	*TULP1**	6p21.3
	*LRAT**	4q31

ar cone-rod dystrophy		
gene	*ABCA4*	1p21-p13
loci	CORD8	1q12-q24
	CORD9	8p11

Stargardt disease		
gene	*ABCA4*	1p21-p13

1.5.3 Characteristics of a selection of retinal dystrophy-associated proteins

ABCA4

The photoreceptor-specific ATP-binding cassette transporter (ABCA4) is a member of the ABC transporter family[50]. The ABCA4 glycoprotein is located at the rim of the outer segment disc membrane (figure 1.3) and functions as an outwardly directed flippase for N-retinylidene-phosphatidylethanolamine[21], transporting all-*trans*-

retinaldehyde from the outer segment disc to the photoreceptor cytoplasm from where it moves out of the cell to the RPE after conversion to all-*trans*-retinol.

AIPL1

The arylhydrocarbon receptor interacting protein-like 1 (AIPL1) is a member of the fatty acid binding protein (FABP) family and possesses a peptidyl-prolyl isomerase (PPI) domain and three consecutive tetratricopeptide motifs (TPR)[51]. AIPL1 has a role in cone development but is surprisingly only detected in adult rod photoreceptors in which AIPL1 functions as a chaperone required for rod cGMP phophodiesterase biosynthesis[14].

CRB1

Crumbs homolog-1 (CRB1) is a transmembrane protein, synthesized in the developing and adult human retina and in human brain. CRB1 is localized specifically to the sub-apical region adjacent to the adherens junction complex at the outer limiting membrane in the retina. CRB1 consists of multiple domains of which the large extracellular domain possibly is involved in cell-cell interaction. A small intracellular domain functions as an anchor for several protein complexes in the cytoplasm and is essential for the maintenance of cell polarity and maintenance of the belt like structure called the outer limiting membrane. Furthermore, CRB1 plays a crucial role in photoreceptor cell morphogenesis[52,53].

CRX

The cone-rod homeobox protein (CRX) is a transcription factor synthesized in both photoreceptors and the pinealocytes of the pineal gland[54]. In the retina the CRX protein is required for the development and maintenance of photoreceptor function by regulating the transcription of many photoreceptor-specific genes[39,54-57]. Mutant mice with defective CRX do not elaborate an OS, and several key phototransduction proteins fail to be expressed[58].

GUCY2D

The guanylate cyclase 2D (GUCY2D, also referred to as RETGC1) transmembrane protein is predominantly present in the outer segments of photoreceptors (**figure 1.2**). It consists of several functional domains, i.e. an extracellular domain, a transmembrane domain, a kinase homologous domain, a dimerisation domain, and a catalytic domain[59]. Mutations in *GUCY2D* prohibit the production of cGMP resulting in the continued closure of the cGMP-gated channels. Once the phototransduction cascade is initiated, mutations in this gene will lead to a state resembling continuous light exposure, since the recovery state cannot be accomplished because of the lack of guanylyl cyclase enzyme.

RDH12

Retinol dehydrogenase 12 (RDH12) belongs to the superfamily of short-chain alcohol dehydrogenases and reductases and is specifically synthesized in

photoreceptor cells. The exact role of RDH12 is not known yet, but it was suggested that RDH12 plays a pivotal role in the formation of 11-*cis*-retinal from 11-*cis*-retinol, during regeneration of cone visual pigments[18].

RPE65

The retinal pigment-specific 65 kDa (RPE65) protein is crucial in the visual cycle in order to regenerate the chromophore 11-*cis*-retinol[27,60]. Mutations in *RPE65* prohibit the isomerohydrolysation of 11-*cis*-retinol resulting in accumulation of all-*trans*-retinal and opsin apoprotein (unliganded opsin). Apoptosis may be initiated by the high levels of unliganded opsin, for they lead to spontaneous activation of the visual cascade and consequently lead to closure of cGMP-gated channels, again suggesting a state resembling continuous light exposure. The subsequent low levels of intracellular calcium may be a trigger for cell death[61].

RPGRIP1

The retinitis pigmentosa GTPase regulator-interacting protein (RPGRIP1) interacts with the RPGR protein and both are localized at the connecting cilia of rods and cones[62]. Mutations in RPGR cause up to 72% of the X-linked RP cases[63], whereas RPGRIP1 mutations are associated with the much more severe LCA. There are numerous isoforms of RPGRIP1, and they seem to have complementary functions in cytoskeletal-mediated processes in photoreceptors and amacrine neurons[64].

1.5.4 Strategies for gene and gene mutation detection

Since the identification of the first retinal disease genes 15 years ago[2,3] a host of retinal genes have been identified. The thus far identified genes harbor mutations in approximately 50% of the patients, which leaves 50% of the molecular causes as yet unknown.

The identification of the underlying genes has had a tremendous impact. It improved our understanding of the function of the normal retina and the pathogenesis of several degenerative diseases. Furthermore, it forms the basis for studies aimed at therapeutic intervention. Unfortunately, molecular diagnostics has not yet made its way into the every day clinical practice, a fact that has much to do with the staggering amount of genetic heterogeneity and its resulting costs and manpower issues.

Several newly developed therapies are currently being tested, some of which have already reached the human clinical trial phase. As a consequence, there is a need for the discovery of the additional 50% of retinal disease causing genes and a need for methods that provide a rapid and accurate screening ability in order to identify the causative genes and their mutations in individual patients.

1.5.5 Whole-genome linkage analysis

The discovery of new disease genes is expensive and time consuming. Through the effort of the human genome project, the human DNA sequence is now completely known, enabling the physical localization of microsatellite markers and single nucleotide polymorphisms (SNP).

In the past 10 years microsatellite markers were traditionally used for linkage studies. They contain tandem repeats of a simple DNA sequence and are often very polymorphic, i.e. consist of many different alleles. In whole-genome scans these markers are spaced at intervals of approximately 10 cM across the genome. This is a labor-intensive method and relatively costly (approximately €12.000 for a genome scan of a family with 20 individuals).

SNPs are only biallelic and therefore are generally less polymorphic than microsatellite markers. On the other hand, SNPs are present more frequently. Their analysis can be carried out using high-density microarrays, which makes it very fast and less expensive (approximately €5.400 for a genome scan of a family with 20 individuals).

1.5.6 Mutation detection

The identification of retinal disease genes gave rise to a demand for rapid and reliable screening tools for genotyping. For several reasons it is important to establish the underlying molecular cause in a patient with a retinal dystrophy. 1. It provides an exact diagnosis, based on the molecular defect and thus is much more accurate than a diagnosis based on retinal appearance, disease onset and progression. This is important because different retinal dystrophies have overlapping symptoms and signs. 2. It provides a tool to give a prognosis for the future visual course, 3. It enables well-founded genetic counseling, 4. It may enable prenatal screening, 5. It aids in determining eligibility for certain therapies, 6. It allows the identification of novel cellular and molecular processes, through the identification of new retinal dystrophy genes.

Sequence analysis of the involved gene is considered the “golden standard” when there is a small number of candidate genes and when the numbers of exons are limited. Exons are part of the nucleotide sequence in DNA that carries the code for the mature mRNA molecule and thus encodes the protein’s amino acid sequence. Alternatively only the exons containing hotspots (a region in a gene in which there is a high occurrence of mutations) for mutations can be sequenced.

When a large number of highly complex genes need to be analyzed, alternative methods are needed. One such a screening tool that serves this demand is the Affymetrix’s GeneChip CustomSeq Resequencing Array (AGRA). This technique uses a four-probe interrogation strategy. For the reference sequence of the coding

region of the genes of interest and small stretches of their flanking intron sequences, four probes (17-mer oligonucleotides) are designed to interrogate a single position. One probe is designed to be perfectly complementary to a stretch of the reference sequence; the other three are identical to the first, except at one position where one of the other three bases are substituted. Sample DNA is amplified, fragmented, fluorescently labeled, purified and hybridized to the designed probes. The probe that forms the most stable duplex will provide the highest fluorescent signal among the four probes assigned to interrogate the central base. The next nucleotide in the target sequence is identically interrogated, using another set of four oligonucleotide probes (figure 1.5). This analysis is carried out for both DNA strands of the double helix. This strategy is repeated in a manner that covers a single continuous sequence of interest. For example, one hundred sets of eight probes are used to interrogate one hundred bases of sequence. Specially designed software (Affymetrix GeneChip DNA Analysis Software) is used to analyze the results. In general this technique detects homozygous and heterozygous base substitutions robustly. Currently, these arrays also test for the presence of one-nucleotide deletions, but not for the presence of other heterozygous abnormalities such as insertions and other deletions (www.affymetrix.com). This is an effective though expensive approach in small families or isolated cases for detecting both known as well as new mutations. For arRP such an array is now available. It contains 17-mers from most of the open reading frame from sequences of 11 out of the currently known 19 arRP genes[65].

A different approach is the arrayed primed extension (APEX) technology (mini-sequencing), in which oligonucleotides, custom-designed to identify previously detected gene mutations, are arrayed on a glass slide. A mini-sequencing reaction is performed using four unique dye-labeled terminator nucleotides and DNA polymerase, followed by stringent washing of the slides. The slides are imaged with the Genorama™ QuattroImager and the sequence variants are identified by Genorama™ genotyping software. The slides can only be used once, and can of course only detect previously identified mutations (figure 1.6). The ABCR500, LCA and Usher syndrome microarrays are currently available, and arRP, adRP and xlRP microarrays are under development (www.asperbio.org). A comparison between the AGRA, APEX and sequence analysis techniques is outlined in table 1.2.

Table 1.2 Comparison of different genotyping techniques for one patient analyzed for defects in 10 genes (with an average of 10 exons)

	AGRA	APEX	Sequence analysis
Advantage	• identifies all small nucleotide substitutions	• rapid • affordable (€100/ sample)	• identifies all small nucleotide changes
Disadvantage	• misses heterozygous insertions and deletions • expensive (€1.500/ sample)	• does not detect new mutations	• time consuming • expensive in larger genes (€1.200/sample)

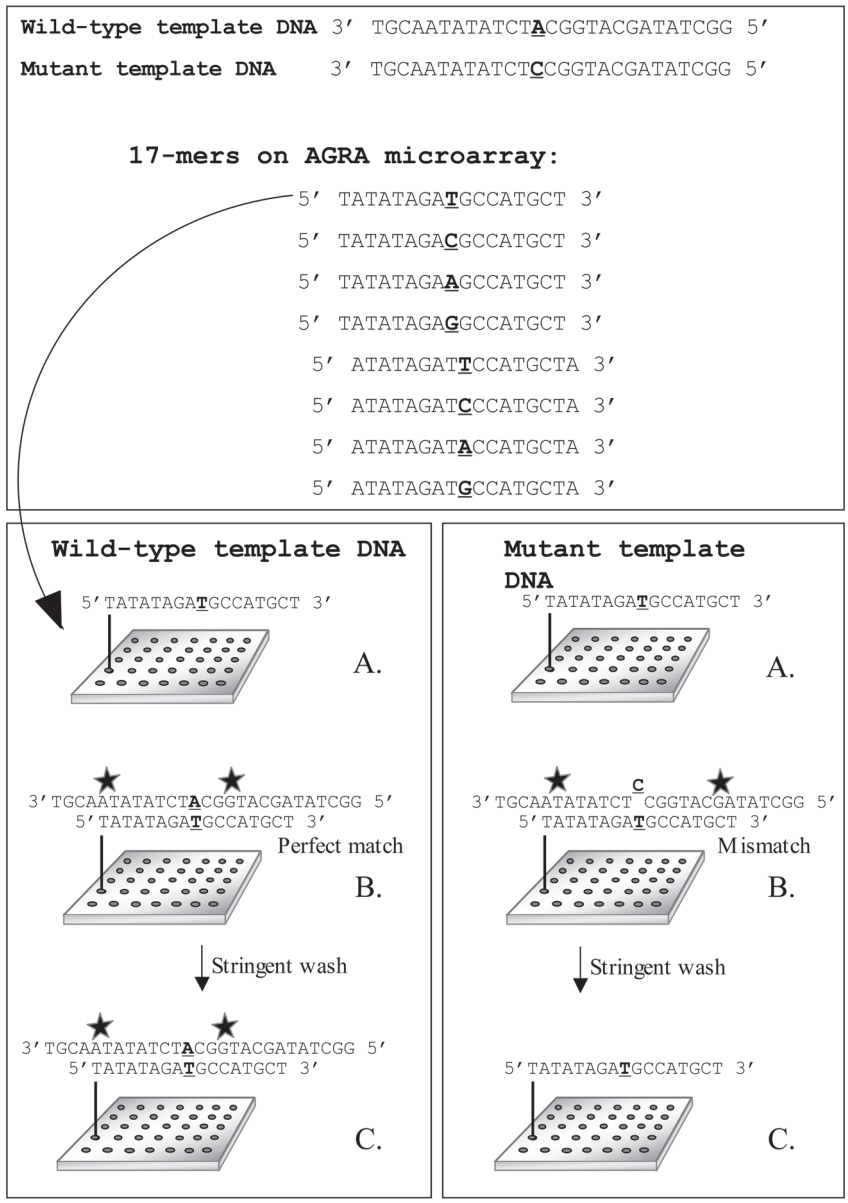

Figure 1.5 Schematic representation of the AGRA technique
The same 17-mer probe hybridisation is depicted for wildtype and mutant template (PCRed genomic) DNA. A. 'Wild-type' 17-mer linked to glass slide. B. Hybridization of wild-type template (perfect match) and mutant template (mismatch) to the 17-mer. C. Upon stringent wash, only the wild-type template remains annealed and a signal is detected.

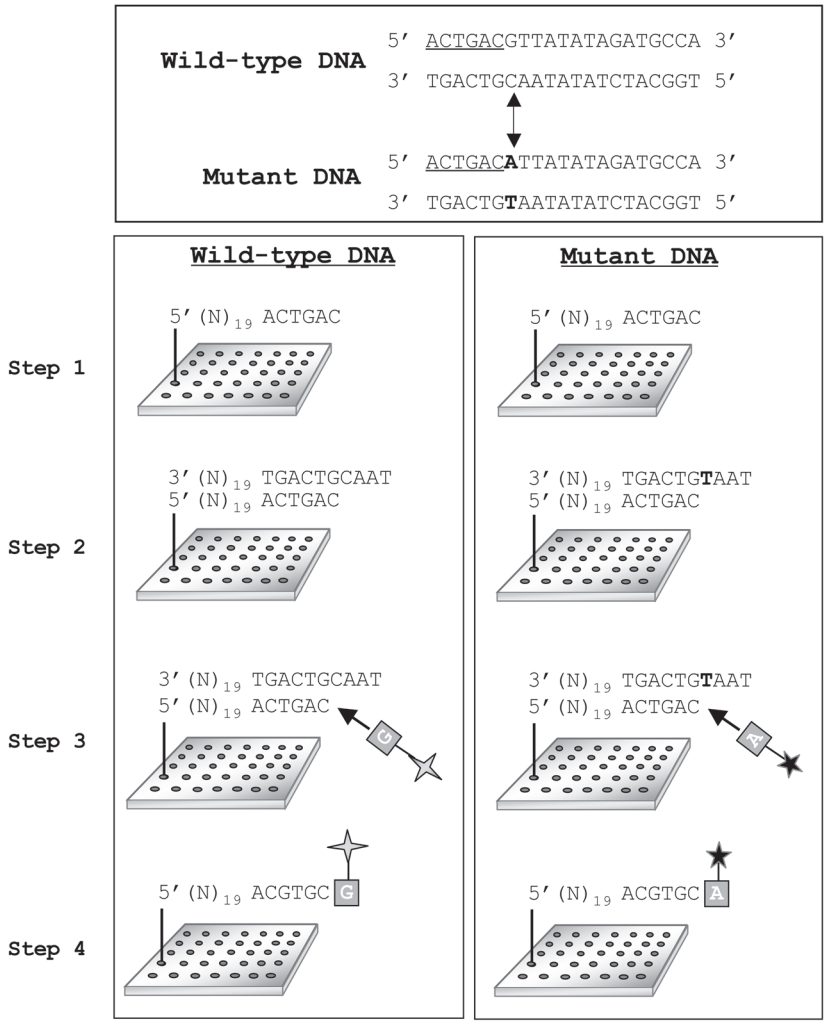

Figure 1.6 Outline of the allele-specific primer extension (APEX) technique

Step 1: 25-mers specific for a nucleotide sequence just next to a variant nucleotide are linked to a glass slide.

Step 2: hybridization of fractionated wild-type or mutant genomic sequences amplified by PCR.

Step 3: primer-extension using fluorescently labeled dideoxynucleotides distinguishes between different variants.

Step 4: separation of template DNA and detection of specific fluorescent dye.

1.6 Therapeutic prospectives

With the exception of LCA patients, who are generally born blind or severely visually impaired, patients suffering from most other inherited retinal dystrophies most commonly slowly lose their remaining visual function. As yet there is no definitive treatment available. This devastating knowledge has led desperate patients to seek controversial "treatments", sometimes with serious adverse events[66]. Currently, three experimental treatments are being tested in human clinical trials after many years of successful animal model studies.

1.6.1 Gene replacement

Gene replacement therapy represents the most logical approach for long-term therapeutic effects in inherited retinal degenerations. In principle, mutations that lead to loss-of-function (as in autosomal recessive and X-linked diseases, but also in autosomal dominant diseases caused by haplo-insufficiency), the genetic defect in principle can be corrected through the introduction of a wild-type version of the gene into the cells in which normal functioning of this gene is required (in most cases photoreceptor cells or the RPE). Replacement is actually not correct since the mutant gene is not removed.

There are many animal models of autosomal recessive retinal diseases, and some studies with these animals have proven great potential. Rpe65-/- canines lack the RPE65 protein involved in the retinoid metabolism. By a single injection of a recombinant adeno-associated virus (AAV) carrying the missing wild-type RPE65 cDNA into the subretinal space, Acland and co-workers obtained definitive recovery of the visual function as was proven objectively by functional tests (ERG, reaching 20% of the normal cone response) and subjectively by behavioral studies and the apparent loss of nystagmus[67,68]. At the time of writing this thesis the first human trials for gene-replacement therapy for retinal disease are about to start. It will take time though, before this technology comes into clinical practice. Moreover, this therapy will not be applicable to all LCA and RP patients, for mutations in *RPE65* have been identified in only a minority (6-16%) of the LCA and juvenile RP patients[48].

1.6.2 Mutation-independent therapeutic strategies

Mutation-independent therapies may play an important role in future treatments of RP, but will theoretically only slow down or stop photoreceptor apoptosis. They will be applicable to most patients regardless of the genetic defect and will limit their handicap. The therapeutic effects of these therapies are based on transferring anti-apoptotic genes to the photoreceptors or, more readily, deliver neuroprotective factors. These modulate the microenvironment of the photoreceptors and by that stabilize the disease before loss of cone photoreceptor cells occurs.

Several trophic factors are used in studies with animal models and all show promising results on delaying photoreceptor degeneration. Unfortunately the serious negative side effects have thus far made these factors unacceptable for human therapy[69-71]. Currently, the most promising trophic factors for treatment of RP are glial cell line-derived neurotrophic factor (GDNF)[72] and brain-derived neurotrophic factor (BDNF)[73]. So far, the precise pathway of action of the neuroprotective factors remains unknown and more fundamental research is necessary.

In 1993 Berson and colleagues demonstrated in a large randomized, controlled, double-masked blind treatment trial with vitamin A palmitate, a small but significant slowing of the retinal disease progression by a daily intake of 15.000 IU[73-75]. In a more recent study Berson demonstrated that docosahexaenoic acid taken in addition to vitamin A had a positive effect on further slowing down disease course[73,76,77]. Caution should be taken since the patient cohort was very heterogeneous. In some cases, depending on the type of molecular defect, accumulation of vitamin A may occur, which as a consequence may result in toxic levels of vitamin A. Furthermore, high doses of vitamin A are teratogenic and should therefore not be subscribed before or during pregnancy.

The sensation of a visual stimulus does not require viable eye tissue since this can also be reached by direct stimulation of the appropriate area of the visual cortex. For this purpose epiretinal devices have been developed that deliver a signal that bypasses the sophisticated coding of the visual system. In RP the photoreceptors are damaged, dead or absent but the inner retina remains relatively healthy. In five patients with bare or no light perception, intraocular placed electrodes coupled with an optically isolated, constant current generator, were used to deliver these pulses and proved to elicit a visual perception of a correct retinotopical spot. In one of the five patients this resulted in a visual acuity of 1/60[73,78]. Ultimately, these artificial retinal chip implants may serve patients with severe generalized retinal degeneration who have little or no perception of light.

1.6.3. RPE and stem cell transplantations

Replacement studies of the RPE, in order to obtain beneficial effects for the adjacent photoreceptors, and of retinal neuronal transplantation, in order to replace the degenerate tissue altogether, were moderately successful in animal models. The ultimate goal of these transplantations is to replace lost tissue with viable and healthy cells that have the capacity to re-establish the cellular connections[73,79]. Currently, it is even possible to transplant embryonic retinal cells to degenerating retinas in animal models. The cells are viable, proliferate, differentiate and are protected by the immune privilege of the eye. Unfortunately, only a small fraction of the transplanted cells survive and the normal retinal structure is never regained[80]. Of course in humans the transplant of embryonic cells faces ethical questions and alternatives are under investigation. The transplantation of healthy RPE cells in the

subretinal space in an animal model proved to delay photoreceptor and retinal function loss[81-84]. Unfortunately this will be of little relevance to most human forms of retinal dystrophy. Radtke and co-workers used a graft of fetal neural retina with its RPE and transplanted this, unilaterally, into the subretinal space under the fovea of an RP patient. After one year there were still no clinical signs of graft rejection and there was an improvement of the visual acuity from 20/800 to 20/160[85].

Recently Tomita[86] and Kicic and co-workers[87] demonstrated the ability of bone marrow-derived stem cells to integrate into the host retinas, to differentiate into cells expressing photoreceptor-specific markers and to establish neuronal connections after transplantation of these cells in vivo. Previous studies that demonstrated stem cell transplantation as a therapy in neurodegenerative diseases were under heavy discussion and faced ethical and legislative opposition for they used allogenic adult brain-derived neural progenitor cells or embryonic retinal precursors. Furthermore, these techniques were of potential risk for infection transmission and potential graft rejection. The new techniques use autologous bone marrow-derived stem cells and so bypasses these oppositions.

In conclusion, worldwide efforts for generating treatments for inherited retinal diseases are promising but are still facing a long way before becoming accessible for humans suffering from inherited retinal dystrophies. A better understanding of the cellular and molecular interactions of photoreceptors and their environment before and during degeneration will not only provide novel information on normal retinal development and function but will also help in understanding disease pathogenesis forming a true basis for identifying treatment possibilities.

1.7 Outline of this thesis

Accurate genotype-phenotype correlation studies of the heterogeneous group of retinal dystrophies will contribute to delineate different and overlapping types of disease and improve the present classifications. A more detailed clinical and genetic classification will help ophthalmologists to give a more accurate diagnosis and enhance the knowledge on disease course. In addition will the clinical appearance help to suggest the underlying molecular defect. Accordingly, accuracy of individual counseling will increase. Structural molecular analysis will also allow prenatal diagnostics in selected cases and in the future, it will help in differentiating patients for different kinds of therapy. Patients in which no molecular defects can be found will also be of interest as they form a patient cohort in which new retinal dystrophy genes can be identified with novel strategies.

There is a growing need for the integration of the clinical characteristics of inherited retinal dystrophies with the molecular diagnosis. The main objective of this

thesis was to contribute to the clarification of genotype-phenotype correlations in autosomal recessive inherited retinal dystrophies. In chapters 2.1 and 2.2 the molecular analysis of mutations in the *ABCA4* gene using two new techniques, and their phenotypical correlation, are discussed. This provided new information on the use and usefulness of rapid molecular screening tools and the phenotypical descriptions of arRP and arCRD patients with mutations in *ABCA4.*

In chapter 3 the different genotypes and phenotypes in Leber congenital amaurosis are discussed showing that mutations in different LCA genes lead to different clinical subtypes of LCA, both clinical and functional.

Two of the LCA genes discussed in chapter 3 are discussed in more detail in chapters 4 and 5. In chapter 4 a large Dutch consanguineous family with homozygous mutations in the *RPE65* gene is described, suggesting that patients with the same mutations do not necessarily display an identical phenotype. In chapter 5, data are presented that show that most heterozygous carriers of *CRB1* mutations display a focal functional abnormality upon multifocal ERG testing predominantly affecting the infero-nasal retina.

Finally, the results of this thesis on autosomal recessive retinal dystrophies is discussed and summarized in chapters 6 and 7 respectively.

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2 Genotype-phenotype analysis of *ABCA4*

2.1

Microarray-based mutation analysis of the *ABCA4 (ABCR)* gene in autosomal recessive cone-rod dystrophy and retinitis pigmentosa

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Abstract

Mutations in the *ABCA4* gene have been associated with autosomal recessive Stargardt disease (STGD1), cone-rod dystrophy (CRD), and retinitis pigmentosa (RP). We employed a recently developed genotyping microarray, the ABCR400 chip, to search for known *ABCA4* mutations in patients with isolated or autosomal recessive CRD (54 cases) or RP (90 cases). We performed detailed ophthalmologic examinations and identified at least one *ABCA4* mutation in 18 patients (33%) with CRD and in 5 patients (5.6%) with RP. Single strand conformation polymorphism (SSCP) analysis and subsequent DNA sequencing revealed four novel missense mutations (R24C, E161K, P597S, G618E) and a novel 1-bp deletion (5888delG) on the second allele of the patients in this cohort. Ophthalmoscopic abnormalities in CRD patients ranged from minor granular pigmentary changes in the posterior pole to widespread atrophy. In 12 patients with recordable electroretinogram (ERG) tracings, a cone-rod pattern was detected. Three patients demonstrated progression from a retinal dystrophy resembling STGD1 to a more widespread degeneration, and subsequently received the diagnosis of CRD. In addition to a variable degree of atrophy, all RP patients displayed ophthalmologic characteristics of classic RP. When detectable, ERG recordings in these patients demonstrated rod-cone patterns of photoreceptor degeneration. In conclusion, in this study we show that the *ABCA4* mutation chip is an efficient first screening tool for autosomal recessive CRD.

Introduction

The *ABCA4* (ABCR) gene was identified as the gene underlying autosomal recessive Stargardt disease (STGD1)[1]. Since the cloning of the *ABCA4* gene, other studies have implicated this gene also in autosomal recessive CRD (arCRD) and in autosomal recessive RP (arRP)[2-17]. In addition, heterozygous *ABCA4* mutations were found in 16% of cases with age-related macular degeneration[18]. The high prevalence of heterozygous *ABCA4* mutations in the general population and the inability of relatively small-sized studies to replicate this result, shed doubt on the significance of the molecular findings in patients with AMD[19-24]. In a larger independent study, two *ABCA4* mutations were significantly associated with AMD[25].

The *ABCA4* gene encodes the ABCR protein, previously identified as the rim protein (RmP), a retina-specific (adenosine triphosphate) ATP-binding cassette transporter. This protein is thought to act as a flippase for *N*-retinylidene phosphatidylethanolamine (N-retinylidene-PE), thereby facilitating the transport of all-*trans*-retinal from the disk lumen to the photoreceptor cytoplasm[26,27]. In *Abcr-/-* mice, N-retinylidene-PE is converted, through various intermediates, into A2E, a major component of lipofuscin. A2E accumulates in toxic levels in the retinal pigment epithelium (RPE), eventually resulting in the degeneration of the RPE and the overlying neuroretina. Interestingly, *Abcr-/-* mice raised in complete darkness do not accumulate A2E, suggesting that avoiding excessive light might be beneficial for humans with *ABCA4*-associated retinal dystrophy[28]. Recently, Radu and colleagues were able to rescue retinal dystrophy in *Abcr-/-* mice by treating them with isotretinoin, a drug commonly used in dermatology for the treatment of severe acne[29]. Both studies show that therapeutic approaches for humans with *ABCA4*-associated retinal dystrophies are feasible, which underlines the importance to identify patients with CRD and RP caused by mutations in *ABCA4*.

Numerous mutation analysis studies in patients with STGD1 have yielded more than 400 different *ABCA4* mutations[1,6,8,9,22,30-35]. Unlike STGD1, arCRD and arRP are not genetically homogeneous disorders (www.sph.uth.tmc.edu/RetNet)[2,36-38]. Studies investigating the role of *ABCA4* mutations in arCRD estimate involvement of this gene in 24-75% of all cases[5-9,11,16]. *ABCA4* mutation analysis in arRP patients thus far was restricted to families in which STGD1 and RP segregated[4,10,14,15,17]. Therefore, it has not been possible to accurately predict the contribution of *ABCA4* mutations in arRP pathology. Recently, a genotyping microarray (ABCR400 chip) was developed containing all currently known *ABCA4* mutations which reliably identifies all known *ABCA4* mutations in patients with STGD1[39].

In this study, we utilized the ABCR400 array to systematically screen for mutations in patients with isolated or autosomal recessive RP (90 patients) and CRD (54 patients). We re-evaluated the clinical features in patients with *ABCA4* mutations and provide, for the first time, an assessment of the contribution of *ABCA4* gene mutations as a cause for arRP in a Caucasian population.

Materials and Methods

Patients and controls

The charts of 90 patients with isolated (56) or autosomal recessive (34) RP and 54 patients with isolated (35) or autosomal recessive (19) CRD were collected from the centres collaborating in this study. RP patients were ascertained in Nijmegen (74), Rotterdam (10), and Heidelberg (6). CRD patients were ascertained in Heidelberg (36), Nijmegen (14), and Rotterdam (4). Some of these cases might be due to X-linked or autosomal dominant mutations. In the remainder of this chapter the patient groups will be designated as CRD and RP.

This study was approved by the institutional review board (CCMO). After informed consent was obtained, blood samples were taken. Samples from 93 healthy Dutch blood donors were used as controls. The clinical data of all patients were examined and, when data were incomplete or obtained with obsolete methods, patients were clinically re-evaluated. Kinetic perimetry was performed with the Goldmann perimeter. A recent electroretinogram (ERG), recorded in accordance with the ISCEV protocol[40], was available for all patients, except for patient 9444. The employed ERG methods for this patient, as well as the earlier ERGs of three other patients, were performed as described by Thijssen et al[41] (patients 12608 and 15680) and Alexandridis et al (patient 15730)[42]. When possible, color vision was tested with the Ishihara and Panel D15 tests. The diagnosis of CRD was based on initial complaints of decreased or blurred central vision, without a history of night blindness. Maculopathy, characterized by a bull's eye pattern or granular alterations of the macular RPE, with or without relatively mild peripheral retinopathy was considered typical. Visual field testing usually shows a central scotoma, while the peripheral fields are either normal or show a mild to moderate constriction. In addition, ERG recordings in CRD either show reduction or absence of cone responses in the presence of quantitatively less reduction in rod responses, or an equal impairment of both photoreceptor systems[43-46]. The initial symptom in RP patients is night blindness. Visual field defects typically originate in the midperiphery, with gradual enlargement to both the periphery and the centre of the retina. Typically, the ERG recordings demonstrate photoreceptor degeneration in a rod-cone pattern[47]. In many cases, especially in the later stages of both CRD and RP, rod and cone ERGs may be equally impaired or may become non-recordable, which makes it difficult to provide an accurate diagnosis. In such cases the nature of the initial complaints, the aspect of the fundus and - if available - ERG recordings of an earlier stage of the retinal dystrophy are used to discriminate between RP and CRD.

Mutation screening

The microarray mutation analysis of the *ABCA4* gene with the ABCR400 chip was performed as described earlier[39]. In patients with one *ABCA4* mutation, we

searched for the second mutation using single strand conformation polymorphism (SSCP) analysis and DNA sequence analysis of aberrantly migrating fragments as described elsewhere[5] (ref 5 and references therein). The ABI PRISM Big Dye Terminator Cycle Sequencing V2.0 Kit was used for sequencing and the reactions were analyzed with the ABI PRISM 3700 DNA analyzer (Applied Biosystems).

Four novel missense mutations were tested in 93 control DNA samples. The presence of p.R24C (c.70C>T) was analyzed using *Hin*fI restriction fragment analysis of PCR-amplified exon 2. The normal PCR product (191 nts) was cut into fragments of 167 and 24 nts; the mutant PCR product with the 70C>T alteration was not digested. p.E161K (c.481G>A) was tested using *Mbo*II which cuts the normal PCR product of exon 5 (240 nts) in three fragments (170, 40, 30 nts) and the mutant PCR product in two fragments (200 and 40 nts). The p.P597S (c.1789C>T) mutation was analyzed using *Alw*I, which cuts the normal PCR product of exon 13 (280 nts) into fragments of 100, 90, 60 and 30 nts and the mutant PCR product into fragments of 160, 90 and 30 nts. An amplification-refractory mutation specific (ARMS) assay[48] was performed to test p.G618E (c.1853G>A). For specific amplification of the mutant sequence, the SSCP reverse primer was used and a mutation-specific forward primer (5' GCAGGACATGGTTGAACAGCA 3'). ARMS cycling parameters consisted of 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, and a final extension of 5 min at 72°C using 2.0 mM $MgCl_2$.

Results

Mutation analysis

Genotyping was performed on DNA isolated from blood samples of 54 CRD patients and 90 RP patients with the ABCR400 chip. Employing the ABCR400 microarray chip we identified *ABCA4* mutations in 18 (33%) patients with CRD (table 2.1.1) and in five (5.6%) patients with RP (table 2.1.2). Eleven of 54 CRD patients were compound heterozygous (9) or homozygous (2) for *ABCA4* variants; seven were heterozygous. Four out of 90 RP patients were heterozygous; one patient was compound heterozygous for *ABCA4* variants. Although the segregation of the p.L541P and p.A1038V mutations could not be tested in the respective families, we have grouped them as complex alleles, based on previous observations that these alterations invariably occur in *cis* configuration in German patients with CRD (table 2.1.1)[5,34]. Indeed, all four patients carrying these variants (14488, 14752, 16242 and 16582) were from Germany. Likewise, the ΔG863 variants are presumed to be located in the same allele since the p.G863A allele in previous studies was always found together with p.R943Q (see discussion)[33,51].

Next, we employed SSCP analysis and DNA sequencing in patients with one *ABCA4* mutation and identified five novel *ABCA4* mutations that were not present on the microarray, that is, p.R24C, p.E161K, p.P597S, p.G618E, and c.5888delG. None of the four new missense mutations could be identified in a panel of 93 healthy control individuals. The c.5888delG mutation is predicted to result in the truncation of the second nucleotide binding domain of the ABCR protein and can thus be considered a null mutation. The four novel missense mutations significantly alter the charge or hydrophobicity of the respective amino acid residues, which invariably are conserved in mouse *Abcr* and, with the exception of p.R24C, also in human *ABCA1* (data not shown). p.R24C is located in the N-terminal cytoplasmic domain, one residue next to the first transmembrane domain of ABCR. p.E161K is located in the first lumenal loop; p.P597S and p.G618E are both located in first cytoplasmic loop.

In three cases with isolated CRD (12608, 16697, and 16887) we were able to perform segregation analysis of the mutations. The mutations in patients 12608 and 16887 were shown to be located on different chromosomes. In family members of patient 16697, the p.G863A; p.R943Q variants segregated from the p.G618E; p.V1433I mutations. For the other patients with two or more *ABCA4* variants, no parents or unaffected siblings were available for genetic analysis. The three variants identified in patient 16243 were arbitrarily indicated in the table since we were unable to test their segregation.

Ophthalmologic analysis of CRD patients

An overview of the clinical findings in the patients with *ABCA4*-associated CRD is given in table 2.1.3. All patients experienced a loss of central visual acuity as the initial symptom; in most patients a central scotoma was present, varying from ten to 40 degrees in size. The visual acuity in most of these patients is 20/125 or lower. An exception is patient 15429 with a visual acuity of 20/32 at 53 years, which cannot be attributed to the early stage of the disease progression. Color vision tests could be performed in 11 patients, who all demonstrated a red-green defect. In ten of the 18 patients, the photopic (cone) responses on the ERG are more severely decreased compared to the scotopic (rod) responses. In patients with nonrecordable or equally reduced cone and rod responses, the diagnosis CRD was based on earlier ERG recordings, the initial symptoms, and the overall aspect of the fundus.

Three patients (14752, 16697, and 16887) presented with yellow flecks, located at the posterior pole and midperiphery (figure 2.1.1b, patient 16887). Two of these individuals were initially diagnosed with STGD1 and demonstrated characteristic blocking of choroidal background fluorescence. However, in the later stage of their disease, these patients developed full-field ERG abnormalities in a cone-rod pattern. These patients should therefore be classified either as STGD1 with peripheral involvement or as CRD.

Table 2.1.1 *ABCA4* sequence variants in CRD patients

Novel variants in bold lettering. *Mutation which is presumed to be in linkage disequilibrium with unknown pathologic *ABCA4* mutation. $polymorphic variants 4203A, 5603T, and 5682C also present. ?, segregation analysis not possible or not determined; NA, not applicable.

CRD patient number	Inheri-tance	Allele 1		Allele 2		Mutations segregate
		Nucleotide change	Effect	Nucleotide change	Effect	
12608	Isolated	IVS38-10T>C	Unknown*	IVS38-10T>C	Unknown*	yes
14488	Isolated	1622T>C; 3113C>T	L541P; A1038V	Not identified		NA
14750	Isolated	4918C>T	R1640W	Not identified		NA
14752	Isolated	1622T>C; 3113C>T	L541P; A1038V	IVS38-10T>C	Unknown*	?
15105	Isolated	IVS36+2T>C	Splicing	IVS40+5G>A	Splicing	?
15428	Isolated	1622T>C; 3113C>T	L541P; A1038V	2300T>A	V767D	?
15429	Isolated	52C>T	R18W	**70C>T**	**R24C**	?
15680	Isolated	5882G>A	G1961E	Not identified		NA
15730	Isolated	2588G>C; 2828G>A	ΔG863/G863A; R943Q	2588G>C; 2828G>A	ΔG863/ G863A; R943Q	?
16242	Isolated	1622T>C; 3113C>T	L541P; A1038V	Not identified		NA
16243	Isolated	5381C>A	A1794D	**1789C>T**	**P597S**	?
		481G>A	**E161K**			
16569	Aut. rec.	3259G>A	E1087K	Not identified		NA
16582	Isolated	1622T>C; 3113C>T	L541P; A1038V	IVS38-10T>C	Unknown*	?
16583	Isolated	194G>A	G65E	768G>T	Splice site	?
16697	Isolated	2588G>C; 2828G>A[$]	ΔG863/G863A; R943Q	**1853G>A;** 4297G>A	**G618E**; V1433I	yes
16755	Isolated	2588G>C; 2828G>A	ΔG863/G863A; R943Q	Not identified		NA
16887	Isolated	768G>T	Splicing	IVS38-10T>C	Unknown*	yes
17906	Aut. rec.	768G>T	Splicing	Not identified		NA

Table 2.1.2 *ABCA4* sequence variants in RP patients

Novel variant in bold lettering. [§]Polymorphic variants 4203A, 5603T, and 5682C also present.

RP patient number	Inheri-tance	Allele 1		Allele 2	
		Nucleotide change	Effect	Nucleotide change	Effect
9304	Aut. Rec.	2588G>C; 2828G>A[§]	ΔG863/G863A; R943Q	**5888delG**	R1963fs
9444	Aut. Rec.	6529G>A	D2177N	Not identified	
9545	Isolated	6529G>A	D2177N	Not identified	
14753	Isolated	1622T>C; 3113C>T	L541P; A1038V	Not identified	
17597	Isolated	6148G>C	V2050L	Not identified	

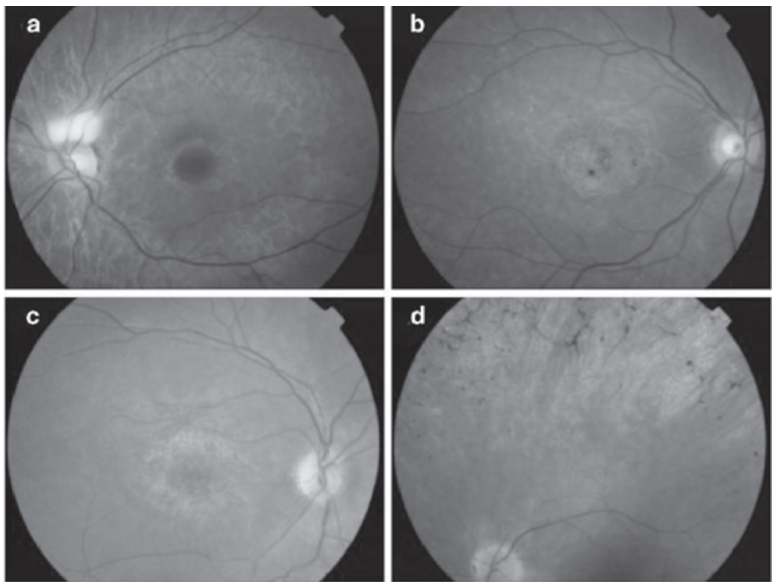

Figure 2.1.1 Fundus pictures of patients with CRD and RP

a. CRD patient 15680 with bull's eye maculopathy and temporal pallor of the optic disc (with myelinated nerve fibers). In the periphery (not visible) there are minor hyperpigmentations but the retinal vessels are of normal size.
b. Chorioretinal atrophy in the posterior pole of CRD patient 16887. Mild granular changes located at mid-peripheral retina (not visible). This patient was initially diagnosed as STGD1, in view of the yellow flecks, which are still faintly visible.
c. CRD patient 16569, taken at age 12, with an obvious pallor of the temporal optic nerve head and atrophic changes in the macula. At that time, the photopic ERG is already nonrecordable and the scotopic ERG is severely decreased.
d. RP patient 17597 shows typical RP features such as bone spiculas in the periphery and attenuated retinal vessels. Large choroidal vessels can be seen in the midperiphery indicative of atrophic changes.

Table 2.1.3 Clinical data of CRD patients with sequence changes identified

CF, counting fingers; HM, hand movements; ND, not detectable; ↓ = decreased; ↓↓ = severely decreased; *, year of examination

No	Sex	Age (yrs)	Visual acuity		Fundus	Perimetry	ERG	
			Right	Left			Photopic	Scotopic
12608	M	40	HM	HM	Peripheral bone spicules and extensive chorioretinal atrophy. Very pale optic disc	Central scotoma of 40 deg.	ND	ND
14488	F	48	CF	20/400	Diffuse, granular RPE changes in macula, peripheral pigmentations	Central scotomas of 25 deg in right eye and 10 deg in left eye.	↓↓	↓
14750	M	17	CF	CF	Central hyper- and hypopigmentation of the RPE	Large paracentral scotomas	ND	ND
14752	F	17	20/400	20/400	Yellow spots in the posterior pole Central RPE changes.	Central scotoma of 15-20 deg.	↓↓/ND	↓
15105	M	25	20/200	20/125	Granular aspect of macular RPE	Central scotoma of 10-15 deg.	↓↓	↓
15428	F	53	CF	CF	Chorioretinal atrophy at the macula with extensive hypo-and hyperpigmentation	Central scotoma of 10-15 deg. Peripheral constriction (50-60 deg).	↓↓	↓
15429	F	53	20/32	20/32	Large central chorioretinal atrophy with small island.	Central scotoma of 15-20 deg.	↓↓	↓
15680	F	51	HM	HM	Chorioretinal atrophy, bull's eye maculopathy	Central scotoma and bad fixation	↓↓ (1985)* ↓↓	↓ (1985)* ↓↓
15730	M	30	CF	CF	Granular RPE changes at the macula	Right eye: only island 20-40 deg in superior field. Left eye: central scotoma of 20 deg.	↓↓ (1985)* ND	↓(1985)* ND

Table 2.1.3 Clinical data of CRD patients with sequence changes identified (continued)

No	Sex	Age (yrs)	Visual acuity		Fundus	Perimetry	ERG	
			Right	Left			Photopic	Scotopic
16242	F	23	20/200	20/200	Bull's eye with central chorioretinal atrophy	Central scotoma and ringscotoma 20-50 deg.	ND	↓↓
16243	M	40	20/125	20/100	Initially granular RPE atrophy at the macula, later geographical atrophy	Central scotoma of 10 deg.	↓↓	↓
16569	M	11	20/400	20/400	Granular RPE changes at the macula. No bone spicules and no attenuated retinal vessels	Large central scotomas.	↓↓/ND	↓↓
16582	F	19	20/400	20/400	Mild granular changes at the macula	Central scotoma of 20 deg.	↓	↓
16583	M	17	20/200	20/400	Granular changes at the macula	Central scotoma of 10-20 deg.	↓↓	↓
16697	F	24	20/125	20/125	Bull's eye maculopathy. Mild flecks at posterior pole	(Para)central scotomas.	↓	↓
16755	F	62	CF	20/400	Granular RPE changes at the macula	Central scotoma of 30-40 deg.	↓↓/ND	↓↓/ND
16887	F	17	20/400	20/400	Central chorioretinal atrophy Granular changes at midperiphery	Central scotoma	↓	↓
17906	F	14	20/63	20/63	Dark pigmentations at the macula, surrounded by hypopigmented halo	(Para)central scotomas	ND	↓

Table 2.1.4 Clinical data of RP patients with sequence changes identified
CF, counting fingers; HM, hand movements; ND, not detectable; ↓, decreased; ↓↓, severely decreased.

No	Sex	Age (yrs)	Visual acuity		Fundus	Perimetry	ERG	
			Right	Left			Photopic	Scotopic
9304	F	87	CF	CF	Severe chorioretinal atrophy	Presently, impossible to perform. Ringscotomas in the past.	ND	ND
9444	M	61	20/63	20/63	Bone spicules and attenuated vessels Minor excavation of disc	Ringscotomas.	↓ (1982) ND	↓↓ (1982) ND
9545	F	60	20/63	20/200	Bone spicules and attenuated vessels Glaucomatous aspect of left optic disc	Initially ringscotomas, later progressive constriction of visual field. Also Bjerrum scotomas, more pronounced in the left eye than in the right eye.	↓	↓
14753	F	25	20/63	20/63	Gliosis at the macula, attenuated retinal vessels	Central island of 10-15 deg. Ringscotoma of 50 deg..	ND	ND
17597	F	48	20/20	20/20	Peripheral bone spicule pigmentations, mild attenuation of vessels, midperipheral atrophy	Partial ringscotoma	↓↓/ND	ND

Ophthalmologic analysis RP patients

The clinical data of the five RP patients with *ABCA4* mutations are summarized in table 2.1.4. Typical RP features like peripheral bone spicules and progressive attenuation of the retinal vasculature were invariably present. Only patient 9304 demonstrated extensive chorioretinal atrophy. Color vision tests could be performed in three patients: all had blue-yellow type defects. In patients 9304 and 14753, no scotopic and photopic ERG responses could be elicited, the other three patients demonstrated a rod-cone pattern of photoreceptor degeneration.

Discussion

In previous *ABCA4* mutation analysis studies, RP patients were ascertained because of their familial relationship with STGD1 patients. In this study, we describe the first systematic search for *ABCA4* mutations in patients with isolated or autosomal recessive RP. In addition, this is the first CRD mutation analysis study that is primarily based on a genotyping microarray. The *ABCA4* gene has shown an extraordinary allelic heterogeneity and most of the sequence variants have been observed in only a few cases. Therefore, the interpretation of the pathologic nature of sequence variants, in particular missense mutations and apparently benign variants, is problematic.

Pathogenicity of *ABCA4* variants

In table 2.1.5, the functional consequences of *ABCA4* missense mutations, that is ABCR protein expression, ATP binding, and ATPase activity, are summarized. Likewise, the known or predicted effects of the splice site mutations are indicated. For two conservative missense mutations (p.V1433I and p.V2050L), the pathologic nature can be questioned. The c.IVS38-10T>C variant is a splice acceptor variant that has no detrimental effect on splicing, but has been found in 27 of 518 STGD1 patients compared to one of 316 ethnically matched control individuals[34,35]. Therefore, it is very well possible that the c.IVS38-10C variants observed in five CRD alleles in our patient cohort are in linkage disequilibrium with an unidentified pathologic *ABCA4* mutation. In two RP patients, we identified the p.D2177N mutation heterozygously. The p.D2177N mutation has never been found in patients with STGD1 but was found to be associated with age-related macular degeneration at a statistically significant level[25]. As shown by Sun et al[50], this mutation, contrary to other mutations, results in increased ATP hydrolysis when compared to the wild type protein[52]. These data do not allow us to draw a definitive conclusion regarding the pathologic nature of p.D2177N.

An unexpected finding is the detection of the ΔG863 variant in four patients; homozygous in one CRD patient and heterozygous in three CRD/RP patients. Based

on a genotype-phenotype correlation model proposed by us and others, individuals carrying two ΔG863 alleles would not be expected to show retinal pathology since this variant was deemed a mild allele[33,49]. In three of the ΔG863 carrying haplotypes (in patients 15730 and 16755, table 2.1.1), the ΔG863 and 2828A variants are not accompanied by the polymorphic variants 4203A, 5603T and 5682C (data not shown), but might have been linked to a more severe mutation in the 3' part of the gene. Also, the ΔG863 variant has been found in *cis* with an intragenic deletion spanning exon 14 of the *ABCA4* gene in a French RP patient[4]. Secondly, other genetic factors might have a significant effect on the phenotypic outcome of *ABCA4* mutations. This was recently demonstrated in one of two siblings with autosomal dominant STGD3-associated macular dystrophy in which a heterozygous *ABCA4* mutation aggravated the retinal dystrophy[52]. Finally, in view of the high carrier frequency of the ΔG863 allele in the Dutch (1.4%)[51] and German (1.0%)[51] populations, patients 15730 and 16755, who only carry the ΔG863 variant(s), might do so by chance.

Table 2.1.5 Functional assessment of missense (A) and splice site (B) mutations

A.

Missense mutation	Nature of amino acid change	Effect on ABCR function (ref.)
R18W	Non-conservative	Unknown
R24C	Non-conservative	Unknown; adjacent to 1st transmembrane domain
G65E	Non-conservative	Unknown
E161K	Non-conservative	Unknown
L541P	Conservative	Decreased ATP-binding & ATPase activity (52)
P597S	Non-conservative	Unknown
G618E	Non-conservative	Unknown
V767D	Non-conservative	Decreased ABCR expression (10)
G863A	Non-conservative	Decreased ATPase activity (52, 53)
R943Q	Non-conservative	Decreased ATPase activity (53)
A1038V	Conservative	Decreased ATP-binding & ATPase activity (52)
E1087K	Non-conservative	Decreased ATP-binding (52)
V1433I	Conservative	Unknown
R1640W	Non-conservative	Unknown
A1794D	Non-conservative	Introduction charged aa in 10th transmembrane domain
G1961E	Non-conservative	Decreased ATP-binding & ATPase activity (52)
V2050L	Conservative	Unknown
D2177N	Non-conservative	Increased ATPase activity (52)

B.

Splice site mutation	Effect on mRNA (ref.)	Predicted effect on ABCR protein
768G>T	Nonsense mediated decay (33)	No protein
IVS36+2T>C	Unknown	Truncation of exon 36 resulting in V1673fs?
IVS38-10T>C	No effect (34)	Variant in linkage disequilibrium with unknown mutation
IVS40+5G>T	350-bp insertion in ~50% of mRNA (34)	Insertion of aberrant amino acid stretch?

Phenotypic spectrum of CRD and RP patients with ABCA4 mutations

CRD patients present with a substantial clinical heterogeneity as observed in other studies[7,13,16,17]. This variability is expressed in the rate of visual loss, the extend of the visual field defects, and the ophthalmoscopic appearance. Three of the 18 *ABCA4*-associated CRD patients in this study represent a subtype that initially resembles STGD1 but, contrary to the classic juvenile macular degeneration of Stargardt, progresses to a more widespread loss of cone and rod photoreceptors. Improved genotype-phenotypes correlations in the future would enable the early detection of STGD1 patients that are at risk for progression to this CRD phenotype.

Thus far, patients with RP and *ABCA4* mutations have demonstrated a remarkably homogeneous phenotype, characterized by severe loss of visual functions, extensive atrophy, and early loss of ERG responses[2-4,10,12,15,17]. In this study, only one of five RP patients with *ABCA4* variants (9304) demonstrates this characteristic atrophy. The RP phenotype in the remaining patients is moderately severe, with variable atrophy; in addition, ERG responses can often still be elicited. Given this clinical presentation and the fact that homozygous null mutations were not found in these patients, it is possible that the *ABCA4* mutations did not contribute to the RP phenotype in some or all of these four patients. If, on the other hand, alterations in the *ABCA4* gene are responsible for these RP phenotypes, the phenotypic variability of *ABCA4*-related RP is higher than assumed.

ABCA4 involvement in CRD and RP patients

Genotyping of 90 RP patients revealed sequence variations in the *ABCA4* gene in five individuals. As discussed above, only one of these patients shows the ophthalmologic features seen in other RP patients with *ABCA4* mutations. Taken into consideration the high heterozygosity frequency of *ABCA4* mutations in the general population, these data strongly suggest that *ABCA4* mutations are only a minor cause (2-5%) of arRP not exceeding the contribution of most other arRP genes (www.sph.uth.tmc.edu/Retnet).

We also identified 27 putative pathologic *ABCA4* alleles in 18 (33%) of 54 patients with CRD. Four additional missense mutations in three of these patients were identified using SSCP and sequence analysis. Besides the *ABCA4* gene, only one other gene (retinol dehydrogenase 5 - *RDH5*) and two loci (CORD8 on 1q12-q24 and CORD9 on 8p11) have been implicated in arCRD or arCD[36-38]. If earlier data are combined with the results of this study, *ABCA4* mutations are found in 40% of the arCRD cases (table 2.1.6). It can be estimated that, on average, the mutation detection efficiency for *ABCA4* mutations is 60% (ref. 39 and references therein), suggesting that *ABCA4* mutations could be present in approximately 67% of arCRD cases.

Table 2.1.6 Incidence of ABCA4 mutations in different cohorts of CRD patients

Study	Number of CRD patients analysed	Number of Patients with *ABCA4* mutations
Maugeri et al[5]	20	13
Papaioannou et al[6]	8	4
Birch et al[7]	30	11
Briggs et al[8]	8	6
Paloma et al[9]	2	2
Ducroq et al[11]	55	13
Fishman et al[16]	30	16
Current study	54	18
Total	207	83 (40%)

Microarray analysis as a tool for DNA diagnostics in CRD and RP

The analysis of the *ABCA4* gene is of importance to establish the mode of inheritance in CRD families, which is associated with very different recurrence risks in the offspring of mutation carriers. In the future, genotyping may also be helpful to accurately predict the development of *ABCA4*-associated retinal dystrophies, especially for the subgroup of patients initially diagnosed as STGD1 with subsequent progression to CRD. In addition, identification of patients with causal *ABCA4* mutations might become very important if novel insights regarding *ABCA4*-associated pathology and treatment of *Abcr-/-* mice develop into rational therapeutics for human patients. It is likely that mutation chip technology, which enables fast, reliable and cost-efficient mutation analysis, will play an important role in these future developments.

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2 Genotype-phenotype analysis of *ABCA4*

2.2

Molecular and phenotypic analysis of a family with autosomal recessive cone-rod dystrophy and Stargardt disease

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Abstract

Purpose

To identify the causative gene mutations in three siblings with severe progressive autosomal recessive cone-rod dystrophy (arCRD) and their fifth paternal cousin with Stargardt disease (STGD1) and to specify the phenotypes.

Methods

A family displaying arCRD and STGD1 was ascertained and screened for mutations using a new microarray (arRP-I) for autosomal recessive retinitis pigmentosa (arRP). All clinical data were evaluated.

Results

We found a new pathologic *ABCA4* splice-site mutation, c.3523-2A>T and the previously reported c.5327C>T (p.P1776L) missense mutation in the arCRD patients. The three siblings shared these two *ABCA4* mutations and showed similar phenotypes. An unusual aspect was nystagmus which was found in one of the arCRD patients. In the STGD1 patient we found the c.5327C>T (p.P1776L) missense mutation and a novel c.868C>T (p.R290W) missense mutation.

Conclusions

Two new *ABCA4* mutations were identified in a family with arCRD and STGD1. A new finding was nystagmus associated with arCRD in one of the patients.

Introduction

Retinal dystrophies display a high degree of clinical and genetic heterogeneity. Frequently, a single disease may be caused by mutations in a multitude of different genes, and in some cases mutations in a single gene may lead to clinically distinct diseases. One such gene is the retina specific ATP-binding cassette transporter (*ABCA4*) gene. Mutations in the *ABCA4* gene have been shown to cause most cases of autosomal recessive Stargardt disease (STGD1) (MIM248200), a significant fraction of cases of autosomal recessive cone-rod dystrophy (arCRD) (MIM604116), and in some cases, mutations in *ABCA4* were found in patients suffering from autosomal recessive retinitis pigmentosa (RP)[1-16]. *ABCA4* has also been suggested to be a susceptibility factor for age-related macular degeneration (AMD)[17,18].

ABCA4 is a member of the ATP-binding cassette (ABC) transporter gene superfamily and encodes the ABCR protein. ABCR is located at the rim of the outer segment discs of rod and cone photoreceptors[19,20] and is involved in the transport of all-*trans*-retinaldehyde across photoreceptor disc membranes from the lumen to the photoreceptor cytoplasm through a flippase activity[21-23]. Mutations in *ABCA4* lead to an accumulation of all-*trans*-retinal inside the photoreceptor disc lumen. This free all-trans-retinal is unfavourable and therefore Schiff-bonded to phosphatidyl ethanolamine. This bondage leads to toxic levels of *N*-retinylidene-*N*-retinylethanolamine (A2E) in the RPE, which results in RPE cell apoptosis, followed by irreversible photoreceptor cell death[23-25].

The variability of severity in the different diseases associated with *ABCA4* mutations has led to a genotype-phenotype model in which the residual activity of the mutated ABCR protein is inversely correlated with the severity of the retinal dystrophy[2-12]. This model predicts that two severe (null) mutations may lead to arRP, a combination of a severe with a moderately severe mutation may result in CRD, and two moderate or a severe and a mild mutation may lead to STGD1[4].

We present a pedigree displaying both arCRD and STGD1 in which some of the causative mutations in *ABCA4* were identified with the Affymetrix Gene Chip® CustomSeq™ Resequencing Array (arRP-I)[26]. This technique allowed us to identify two novel *ABCA4* mutations. Further, we present the *ABCA4*-associated CRD and STGD1 phenotypes and present a new clinical feature, nystagmus.

Methods and patients

This study was approved by the Institutional Review Board of the Montreal Children's Hospital in Montreal and the used protocol adhered to the declaration of Helsinki. After years of follow-up of a family with three siblings suffering from arCRD, all family

members were asked to participate in this study. Informed consents were obtained and blood samples were taken.

Methods

DNA was isolated using the Qiagen DNA isolation kit. DNA samples were then analysed using the arRP-I array. This newly developed custom designed array contain oligonucleotides designed from the exons and 5 bps of flanking intronic sequences from 11 of the 19 currently known arRP genes; *ABCA4*, *CNGA1*, *CRB1*, *MERTK*, *PDE6A, PDE6B, RGR*, *RHO*, *RLBP1*, *RPE65*, and *TULP1*[26]. DNA of patient VI-1 was sequenced bidirectionally for all coding exons using gene-specific PCR primers as described elsewhere (ref. 26 and references therein). The PCR products were purified using the Millipore purification system and thereafter analyzed on ABI3730 or ABI3100 DNA analyzers. Automatic analysis was done by ABI basecaller.

The c.868C>T (p.R290W) sequence change was tested in DNAs from 92 anonymous healthy Dutch individuals and 95 healthy individuals from the Island of São Miguel (Azores, Portugal), by amplifying exon 8 of the *ABCA4* gene, followed by restriction fragment length polymorphism (RFLP) analysis using EagI. The amplicon consists of 400 base pairs (bp), which in case of a wild-type allele will be cut by EagI into fragments of 80 and 320 bp fragments. *EagI* will not cut the mutated allele.

Patients

The pedigree consisted of 3 siblings affected with arCRD and a fifth paternal cousin affected with STGD1 (figure 2.2.1). Genealogic studies revealed that IV:1, IV:2, V:7, and V:8 originated from Sao Miguel, which is part of the Azore Archipelago. All clinical data were analysed retrospectively and additional information was collected through ophthalmic examination including best corrected visual acuity, slit-lamp examination, fundoscopy, electroretinography (ERG), and Goldmann perimetry.

Results

In patient V:6 several likely benign sequence variants in the *ABCA4* gene were identified with the arRP-I chip: c.141A>G (p.P47P), c.1268A>G (p.H423R), c.5603A>T (p.N1868I), c.5682C>G (p.L1894L), c.6069T>C (p.I2023I) and c.4203C>A (p.P1401L). In addition, two likely pathologic variants were identified in *ABCA4*, c.3523-2A>T and c.5327C>T (p.P1776L). Additional sequence analysis was performed to confirm the presence of these two mutations and several intronic sequence changes were then identified (c.302+26A>G, c.859-32T>C, c.1239+18C>A, c.1239+28C>A, c.1356+11delG, c.4352+54A>G, c.5585-51Adel

and c.6817-49C>G). Indeed, sequence analysis confirmed the presence off the two likely pathologic variants.

Sequence analysis of V:2 and V:5 revealed the same mutations. The three unaffected siblings and the mother of the patients only carried the c.3523-2A>T mutation. Further investigation of the family history revealed a fifth paternal cousin with STGD1. Direct sequence analysis of the DNA of patient VI:1 identified the c.5327C>T (p.P1776L) missense mutation and revealed a novel *ABCA4* sequence change, c.868C>T (p.R290W). The c.868C>T (p.R290W) sequence change was not detected in DNA of 92 healthy Dutch controls and in DNAs of 95 healthy individuals from the island of São Miguel.

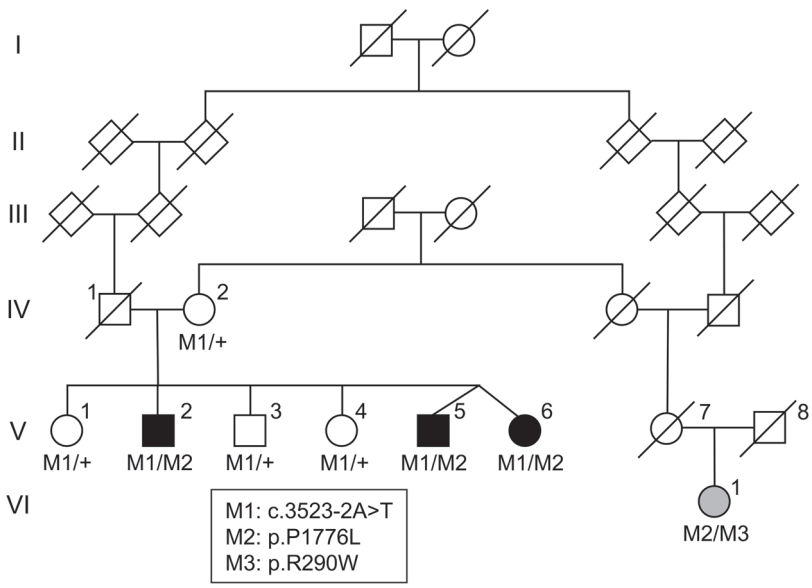

Figure 2.2.1 Pedigree and ABCA4 sequence variants
/ = deceased, □ = male ○ = female, black = affected with arCRD, open = unaffected, shaded circle = affected with STGD1.

Clinical evaluation

Unfortunately, no early clinical data were available for our CRD patients, as the affected siblings with the CRD visited our ocular genetics clinic for the first time in their early forties. On history however, all sibs reported visual acuity difficulties since early childhood followed by peripheral field loss in the second decade. Problems at night occurred in the third decade.

The visual acuity at their first visit to us ranged from light perception to counting fingers. Patient V:2 showed distinct pendular nystagmus. Anterior segments were normal in all three patients. Funduscopy revealed pale optic discs with severely attenuated retinal vessels in all three patients. Individuals V:2 and V:5 showed distinct atrophy of the RPE in the macular area (figures 2.2.2 and 2.2.3). Bone spicule pigmentations were seen throughout the retina in V:2, and limited to the posterior pole and midperiphery in patient V:5. Patient V:6 showed heavy bone spicule pigmentation throughout the entire retina with extensive macular involvement. On ERG, no detectable signals were found in all three patients. Goldmann kinetic perimetry revealed small temporal islands with target V4-e in the arCRD patients.

Patient VI:1 was diagnosed with STGD1 at the age of 11 years at another institution. At 52 years, VA was counting fingers in the right and hand movements in the left eye. Funduscopy revealed normal optic discs, mild attenuation of the vessels and large atrophic lesions in both maculae. In the mid and periphery lobular atrophy of the RPE was seen (figure 2.2.4).

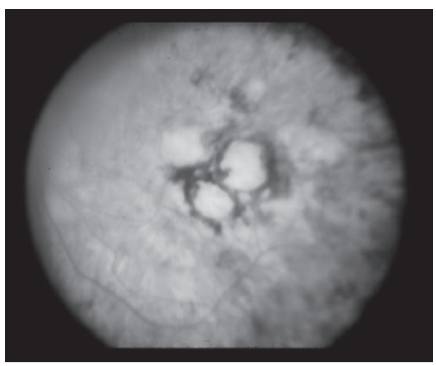

Figure 2.2.2 Fundus photograph of the left eye of patient V:2 (age 46 years)
Note the attenuated vessels, the atrophic lesion in the macula, and bone-spicule pigmentations.

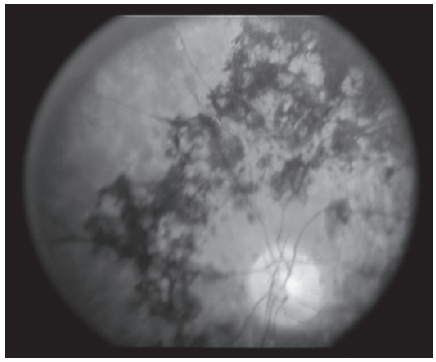

Figure 2.2.3 Fundus photograph of the right eye of patient V:5 (age 43 years)
Note the pale optic disc, moderate attenuation of the vessels and heavy bone-spicule pigmentation in the midperiphery with a relatively spared periphery.

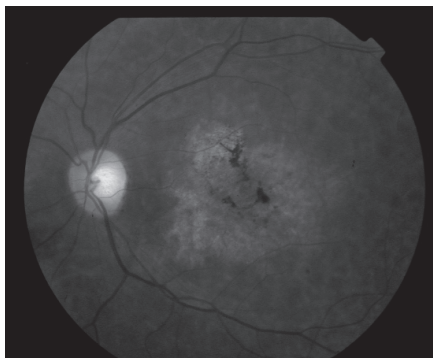

Figure 2.2.4 Fundus photograph of the left eye of patient VI:1 (age 52)
Note the relatively normal optic disc, mild attenuation of the vessels and large atrophic lesion with scattered pigmentations in the macula. The remaining RPE has a lobular atrophic appearance.

Discussion

A family with arCRD and STGD1 was investigated using a new arRP-I array designed to detect mutations in 11 autosomal recessive RP genes including *ABCA4*. In hindsight, the use of the arRP-I chip in this particular pedigree was not logical given the indication of *ABCA4* involvement through the ascertainment of the fifth paternal cousin with STGD1. An alternative much cheaper technique, arrayed primer extension (APEX)-based analysis of the known *ABCA4* variants (ABCR500) could have been used instead. The ABCR500 array would also have identified one of the alleles in both branches of the pedigree.

In three siblings with arCRD both mutations in the *ABCA4* gene were found, i.e. the c.5327C>T (p.P1776L) mutation previously described in a STGD1 patient[10] and a

new splice site mutation; c.3523-2A>T. Direct sequencing of DNA of a fifth paternal cousin with STGD1 from Bermuda (but of Sao Miguel Island origin) revealed the c.5327C>T (p.P1776L) mutation and a new variant, c.868C>T (p.R290W). The arginine at position 290 resides in the first intradiscal loop of ABCR and is conserved in man, mouse, rat, dog and Xenopus. The change from a basic to neutral/hydrophobic residue is likely to have functional implications. The proline residue at position 1776 resides in the middle of a stretch of hydrophobic residues constituting the 9th transmembrane domain of ABCR.

Biochemical analysis of recombinant ABCR bearing these mutations was not performed. Given the previously presented model in which the residual ABCR protein activity is inversely correlated to disease severity suggests that the previously identified p.P1776L[10] mutation is a mild or moderately severe mutation, since both the arCRD and STGD1 sibs shared this mutation. Difference in phenotype would have to be explained by the difference in severity of the c.3523-2A>T (splice site mutation) and the p.R290W (missense) mutations. Most likely, this splice acceptor site mutation preceding exon 24 of *ABCA4* results in the skipping of exon 24, which leads to a frameshift and a translational stopmutation in the third triplet following the exon 23/exon 25 splice junction.

It was difficult to determine the exact clinical diagnosis (especially the issue of RP versus CRD) in our three patients as no early ERGs were available. The occurrence of nystagmus, which is a new finding in a CRD patient with *ABCA4* mutations, supports the history of early loss of central vision. The loss of visual acuity, followed by night blindness and peripheral field loss are suggestive for the diagnosis CRD. In our three siblings the retinal degeneration led to complete loss of the central retina and almost complete loss of the peripheral retina with an RP-like appearance at the age of 40 years. This is consistent with the results from Lorenz and Preising, who suggested that RP caused by *ABCA4* mutations is in fact a severe progressive cone-rod disease[15].

In our STGD1 patient there seemed to be some peripheral involvement as can be expected since *ABCA4* is expressed in both cones and rods. There is no ERG available for this patient, but one might assume that an ERG would also show a cone-rod pattern as in a significant fraction of STGD patients[27].
Several studies are ongoing to design new treatment strategies for retinal dystrophies, some of which are specific for retinal diseases caused by *ABCA4* mutations. Studies with administration of isotretoin and N-(4-hydroxyphenyl)retinamide (HPR) to Abcr -/- mice showed reduction of accumulation of the toxic lipofuscin fluorophores[28,29].

Given these developments, it is important to identify patients with *ABCA4* mutations, as they may be eligible for therapeutic interventions in the near future. Detailed clinical description of these types of retinal dystrophy patients is essential in order to facilitate the search for the causal gene.

In conclusion, mutations in the *ABCA4* gene should be considered in patients with arCRD and in older patients presenting with a severe RP-like phenotype with a history of early central visual acuity loss.

Acknowledgments

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3 Microarray-based mutation detection and phenotypic characterization of patients with Leber congenital amaurosis

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Abstract

Purpose

To test the efficiency of a microarray chip as a diagnostic tool in a cohort of northwestern European patients with Leber congenital amaurosis (LCA) and to perform a genotype-phenotype analysis in patients in whom pathologic mutations were identified.

Methods

DNAs from 58 patients with LCA were analyzed using a microarray chip containing previously identified disease-associated sequence variants in six LCA genes. Mutations identified by chip analysis were confirmed by sequence analysis. On identification of one mutation, all protein coding exons of the relevant genes were sequenced. In addition, sequence analysis of the *RDH12* gene was performed in 22 patients. Patients with mutations were phenotyped.

Results

Pathogenic mutations were identified in 19 of the 58 patients with LCA (32.8%). Four novel sequence variants were identified. Mutations were most frequently found in *CRB1* (15.5%), followed by *GUCY2D* (10.3%). The p.R768W mutation was found in eigth of 10 *GUCY2D* alleles, suggesting that it is a founder mutation in the northwest of Europe. In early childhood, patients with *AIPL1* or *GUCY2D* mutations show essentially normal fundi. In our series, those patients with *AIPL1*-associated LCA progress to an RP-like fundus before the age of eight, whereas patients with *GUCY2D*-associated LCA still have relatively normal fundi in their mid-20s. Patients with *CRB1* mutations present with distinct fundus abnormalities at birth and consistently show characteristics of RP12. Pathogenic *GUCY2D* mutations result in the most severe form of LCA in our series.

Conclusions

Microarray-based mutation detection allowed the identification of 32% of LCA sequence variants and represents an efficient first-pass screening tool. Mutations in *CRB1*, and to a lesser extent, in *GUCY2D*, underlie most LCA cases in this cohort. The present study establishes a genotype-phenotype correlation for *AIPL1*, *CRB1* and *GUCY2D*.

Introduction

In 1869, Theodor Leber reported an “intrauterine” form of retinitis pigmentosa (RP)[1]. These children, mostly from consanguineous marriages, were congenitally blind without any retinal abnormalities. Later in life, many displayed a fundus picture typically found in other retinal dystrophies such as RP. This condition is now known as Leber congenital amaurosis (LCA), a clinically and genetically heterogeneous disorder characterized by severe bilateral congenital retinal blindness, with nystagmus and a nondetectable electroretinogram (ERG) before the age of 1 year[2] .

Thus far, mutations in seven genes (*AIPL1, CRB1, CRX, GUCY2D, RDH12, RPE65* and *RPGRIP1*) have been shown to cause LCA, accounting for 35% to 47.5% of cases of LCA[3-12]. Mutations in all these genes, except *RPGRIP1*, have also been found to cause other, clinically less severe retinal diseases, such as cone-rod dystrophy (CRD) or RP, with autosomal dominant inheritance shown in several cases (ref. 13,14 and references therein.)

Mutations in the *CRB1* gene can cause either LCA or an early-onset form of retinitis pigmentosa, the latter usually refered to as RP12. The clinical characteristics of these autosomal recessive diseases include early central and peripheral visual loss, hyperopia, maculopathy, nummular rather than spicular pigmentation, and limited attenuation of vessels and para-arteriolar preservation of the RPE (PPRPE). Patients occasionally have a Coats-like exudative vasculopathy[3,4].

At present, no treatment is available for LCA or allied retinal dystrophies. However, several treatment strategies are under study, and some have reached the human clinical trial phase[15-19]. Although all other potential treatments would also benefit from more profound knowledge of the underlying genetic defects and disease mechanisms, this is especially true for gene replacement therapy. Although the identification of the causal mutations in known LCA genes is technically not difficult (e.g. using sequence analysis), the genetic heterogeneity and complexity of several LCA genes has hampered their routine molecular analysis. For this purpose, high-throughput techniques are necessary. One such technique, allele-specific primer extension (APEX), has been used in conjunction with a genotyping microarray chip to detect mutations in the *ABCA4* gene. Mutations in this gene are associated with Stargardt disease, autosomal recessive cone-rod dystrophy, autosomal recessive rod-cone dystrophy, and possibly age-related macular degeneration[20-22]. This chip has been a robust and cost-effective screening tool[23,24]. A similar microarray chip was recently developed for genotyping ~300 known mutations in six LCA genes[5].

In this study, we ascertained 58 unrelated patients with LCA, who predominantly live in Belgium and the Netherlands, and we identified the molecular causes in one third of the cases by means of the microarray chip. Clinical data of patients with mutations in the known LCA genes were analyzed retrospectively and all but three were re-examined for the purpose of this study to establish genotype-phenotype correlations.

Patients and methods

Patients

DNA samples from 58 unrelated white patients with a clinical diagnosis of LCA were collected from Belgium (36 cases; Ghent University Hospital, Ghent; University Hospital Leuven, Leuven), The Netherlands (20 cases; Rotterdam Eye Hospital, Rotterdam; Sensis International, Grave; Radboud University Nijmegen Medical Centre, Nijmegen), and Germany (2 cases; University Hospital Heidelberg, Heidelberg; University School of Medicine Berlin, Berlin). All procedures were approved by the ethics boards of the collaborating institutes and adhered to the tenets of the Declaration of Helsinki. All samples were acquired after written informed consent was obtained from the patient or in case of children, their legal guardians. LCA was defined as bilateral visual loss noticed before the age of 6 months, accompanied by wandering nystagmus, and an undetectable or significantly reduced electroretinogram (ERG). Only patients who had not been genotyped previously were included in this study. Patients with a juvenile onset RP (onset after 1 year of age) or a syndromic form of LCA, such as Joubert syndrome, were excluded.

Microarray mutation analysis

Standard protocols were used to extract DNA from peripheral blood leukocytes[25]. The LCA microarray chip contained sense and antisense oligonucleotides (25-mers) corresponding to 301 previously identified disease-associated sequence variants in six of seven currently known LCA genes: *AIPL1* (25 sequence variants), *CRB1* (68 sequence variants), *CRX* (29 sequence variants), *GUCY2D* (66 sequence variants), *RPE65* (81 sequence variants), and *RPGRIP1* (32 sequence variants)[26]. In addition, the chip contained disease-associated sequence variants in two early-onset RP genes: *LRAT* (two sequence variants) and *MERTK* (three sequence variants).

In brief, 68 DNA fragments containing the 301 sequence variants were amplified, and PCR-products were visualized on agarose gels. The PCR products were pooled, denatured and hybridized to the microarray chips. The sequence variants were detected by a sequence-specific primer extension using dideoxynucleotides. For technical details regarding the allele-specific primer extension (APEX) method, see Jaakson et al (ref. 23 and references therein). The results obtained from the LCA microarray chip were confirmed through bidirectional sequencing of the relevant amplicons (ABI3730 and ABI3100 Genetic Analyzers; Applied Biosystems, Inc. [ABI] Foster City, CA). Automatic analysis was performed by a basecaller (ABI).

At the time of mutation chip analysis, *RDH12* had not yet been identified. We therefore sequenced the 7 protein coding exons of *RDH12* in all Dutch and German patients[27].

In those patients in whom one mutation was identified, the entire gene was sequenced. Primers and PCR conditions have been described[3,9,10,28]. Four novel

sequence changes were identified. One of them was a new *GUCY2D* (c.2837C>T; p.A946V) missense mutation that was subsequently tested in DNA from 93 anonymous healthy Dutch control individuals by restriction fragment length polymorphism (RFLP) analysis. *Bst*UI cuts the normal but not the mutant PCR product containing exon 15 (288 bp) in 154 bp and 134 bp fragments. When parents or unaffected siblings were available, segregation analysis was performed.

Phenotyping

In patients with presumed pathologic sequence variants, clinical data were retrospectively collected. In most cases, follow-up clinical information was available, and we used the data from the first and last examinations. At the first visit, parents and patients were questioned about the age of onset of symptoms such as night blindness, photophobia, and photoattraction. A family history was taken and questions concerning pregnancy, birth, birth defects, and consanguinity were asked. Pupillary reactions were observed, and nystagmus and photophobia were evaluated.

Ophthalmic examination included best-corrected, age-appropriate visual acuity testing and measurement of objective refractive error after cycloplegia. The anterior segment was examined by slit lamp biomicroscopy followed by fundoscopy after pupillary dilation. Goldmann visual field results were obtained when possible. ERGs made according to pediatric protocols were obtained in all patients but three at the collaborating institutes. Color vision was tested in four patients with a low-vision edition of the Panel D-15 test (large surfaces) under bright daylight illumination (3500 lux; color temperature of approximately 4500 K), higher than levels normally used in color vision testing (1600 -1900 lux; color temperature of 6500 K)[29]. Images of the fundus were acquired with a fundus photography system (Topcon, Tokyo, Japan). Interpretable autofluorescence images were obtained in two patients with the same system. Interpretable autofluorescence and red free images were acquired with the Heidelberg Retinal Angiograph (HRA2; Heidelberg Engineering, Heidelberg, Germany) in one patient.

Results

Genotypes

The molecular genetic results are summarized in table 3.1. The microarray chip allowed the identification of homozygous or compound heterozygous sequence variants in 14 of 58 patients and single heterozygous sequence variants in an additional six of 58. All variants were confirmed by using sequence analysis of the relevant exons. In the six heterozygous patients, the protein-encoding exons of the

respective genes were sequenced in an attempt to identify the second mutant allele, and four out of the six were identified and were found to be novel sequence variants.

Phenotypes

All available clinical data of the first visit as well as fundoscopic findings at the last examination are summarized in table 3.2. In the following sections, a summary of relevant clinical information is provided. All patients had an ERG performed before the age of 12 months, which was nonrecordable in all except patients 21394, 24105 and 246V1, in whom the ERG was severely reduced.

AIPL1

Genotypes. All three patients with mutations in *AIPL1* were homozygous for p.R278W.

Phenotypes. Over a 15-year period, the visual acuity of patient 14962 remained stable. When he was initially examined during his first year of life because of nystagmus, fundoscopy was normal. At the age of 15, a bull's eye maculopathy as well as increased pigment spicules in the periphery were observed.

Patient 20143 was first examined after he failed to fix and follow at the age of three months. He was mentally handicapped, and regularly poked his eyes (oculodigital sign) and had weak and slow pupillary reactions. At the age of 7.5 years, visual acuity (VA) was 0.003 for both eyes (BE) and the lenses showed a nuclear opacity. Funduscopy revealed a pale optic disc and attenuation of the retinal vasculature, with neither obvious maculopathy nor peripheral intraretinal pigmentation.

When patient 2040V1 was first seen at the age of five months because of nystagmus, fundoscopy revealed diffuse alterations of the retinal pigment epithelium and mild attenuation of vessels. She was night blind and preferred a well-lit environment. At eight years of age, she displayed nystagmus with a predominant slow horizontal component. Best corrected visual acuity was 0.02 with a refraction of +7.5 BE. Axial length was 19.6 mm RE and 19.9 mm LE. On fundoscopy, a bull's eye maculopathy was present with diffuse RPE alterations with only limited spicular intraretinal pigmentation (figure 3.1a). Hyperfluorescent spots were seen on autofluorescence imaging (figure 3.1b).

Table 3.1 Mutations identified in patients with LCA and results from segregation analysis

Bold sequence variants: novel pathogenic mutations identified by sequence analysis. '°', consanguineous parents; '-', no second allele found; aa, amino acid; na, not applicable; nucl., nucleotide; nt, not tested.

Gene	Patient	Variant 1			Variant 2			Segregation of mutations
		Exon	Nucl. change	aa change	Exon	Nucl. change	aa change	
AIPL1	14962	6	c.834G>A	p.W278X	6	c.834G>A	p.W278X	nt
	20143	6	c.834G>A	p.W278X	6	c.834G>A	p.W278X	yes*
	2040V1	6	c.834G>A	p.W278X	6	c.834G>A	p.W278X	yes
CRB1	21394	9	c.2843G>A	p.C948Y	**9**	**c.3664C>T**	**p.Q1222X**	yes
	21405	9	c.2843G>A	p.C948Y	7	c.2234C>T	p.T745M	yes
	21551	9	c.2843G>A	p.C948Y	8	c.2842+5G>A	splice defect	yes
	21554	9	c.2843G>A	p.C948Y	8	c.2842+5G>A	splice defect	yes
	21669	9	c.2843G>A	p.C948Y	7	c.2234C>T	p.T745M	yes
	246V1	7	c.2401A>T	p.K801X	5	c.1084C>T	p.Q362X	nt
	278V1	7	c.2401A>T	p.K801X	8	c.2688T>A	p.C896X	nt
	2148V1	7	c.2401A>T	p.K801X	8	c.2688T>A	p.C896X	yes
	3460V1	7	c.2401A>T	p.K801X	-	-	-	na
GUCY2D	441	8	c.1694T>C	p.F565S	12	c.2302C>T	p.R768W	yes
	20955	12	c.2302C>T	p.R768W	12	c.2302C>T	p.R768W	yes
	21067	12	c.2302C>T	p.R768W	**15**	**c.2837C>T**	**p.A946V**	yes*
	21557	12	c.2302C>T	p.R768W	12	c.2302C>T	p.R768W	yes
	22018	12	c.2302C>T	p.R768W	12	c.2302C>T	p.R768W	nt
	22597	8	c.1694T>C	p.F565S	8	c.1694T>C	p.F565S	nt*
RPE65	23207	5	c.370C>T	p.R124X	**14**	**c.1590delC**	**p.F530fs**	yes

Table 3.2 Ophthalmologic findings in the 19 patients carrying pathogenic mutations

?, no data available; '+', present; '-', absent; absent; HM, hand motion; LP, light perception; m, meter; mo, months; NLP, no light perception; NR, non recordable; OD, right eye; OS, left eye; PPRPE, para-arteriolar preservation of the retinal pigment epithelium; RPE, retinal pigment epithelium; SE, spherical equivalent; yrs, years.

				Early examination after diagnosis										Most recent examination
Gene mutated	Patient number	Noticed at (months)	First symptom	Age	Night blind	Photo-pho-bia	Nys-tag-mus	Visual acuity		Refraction (SE)		Kerato-conus	Fundus	Fundus (age)
								RE	LE	RE	LE			
AIPL1	14962	birth	nystagmus	9 mo	-	+	+	0.002	0.001	+2.5	+3	-	normal	macular RPE alterations in a bull's eye pattern with spicular pigmentations in the periphery (14 yrs)
	20143	3	not following light	14 mo	+	-	+	LP		+10.5	+11.75	+	normal	pale optic disc; narrowing of the arterioles; absent foveal reflex but no clear macular abnormalities; no intraretinal pigment migration (6 yrs)
	2040V1	5	nystagmus	10 mo	+	+	+	LP		+6	+6	-	mild diffuse RPE alterations; attenuation of retinal vessels	macular RPE alterations in a bull's eye pattern, diffuse RPE alterations with scarce intraretinal pigment migration with spicular aspect; sub- or deep intraretinal fine white deposits predominantly along vascular arcades (9 yrs)
CRB1	21394	birth	torticollis	2 yrs	+	-	+	0.05	0.05	+5.5	+5.5	-	normal	pink optic disc; mild narrowing of arterioles; macula shows an atrophic aspect with round, nummular subretinal pigmentations; retinal periphery shows extensive RPE alterations with some intraretinal pigment migration (6 yrs)
	21405	<12	night blindness	3 yrs	+	-	+	0.17	0.20	+4.5	+5.5	-	optic disc normal, normal vessels, subfoveal atrophy of the RPE, peri-pheral pigment alterations	pink optic disc with edema and peri-vascular sheathing; mild narrowing of the arterioles showing tortuositas with subretinal white dots along arterioles with PPRPE; macular aplasia with edema and a few intraretinal hemorrhages; atrophic RPE in periphery with PPRPE, nummular and a few spicular pigmentations (14 yrs)

Table 3.2 Ophthalmologic findings in the 19 patients carrying pathogenic mutations (continued)

?, no data available; '+', present; '-', absent; absent; HM, hand motion; LP, light perception; m, meter; mo, months; NLP, no light perception; NR, non recordable; OD, right eye; OS, left eye; PPRPE, para-arteriolar preservation of the retinal pigment epithelium; RPE, retinal pigment epithelium; SE, spherical equivalent; yrs, years.

				Early examination after diagnosis										Most recent examination
Gene mutated	Patient number	Noticed at (months)	First symptom	Age	Night blind	Photo-pho-bia	Nys-tag-mus	Visual acuity		Refraction (SE)		Kerato-conus	Fundus	Fundus (age)
								RE	LE	RE	LE			
CRB1	21551	4	no eye contact	5 mo	+	?	+	0.07	0.07	+9	+9	?	?	?
	21554	2	oculodigital sign	3 yrs	+	+	+	0.04	0.05	+6	+6	-	macular dysplasia	edematous optic discs with preretinal fibrosis and perivascular fibrotic sheathing without PPRPE; macular dysplasic lesions with progressive outer retinal atrophy of nummular areas temporal to atrophic regions; in mid- and far periphery fine nummular intraretinal hyperpigmentation with fine white deep intra- or subretinal deposits (12yrs)
	21669	6	strabismus	9 mo	?	?	+	LP	0.05	+9.75	+11	?	?	optic disc pale; no PPRPE; macula multiple nummular pigmentations; Coats-like exudative vasculopathy (10 yrs)
	246V1	4	nystagmus	8 yrs	+	+	+	0.01	0.01	+8.5	+7.5	-	absent foveal reflex, attenuation of the retinal vessels, RPE alterations	optic discs are fairly normal; with limited vascular attenuation with relative PPRPE; nummular hyperpigmentation of the macula, retinal periphery and multiple typical white subretinal deposits scattered throughout the fundus (31 yrs)
	278V1	<12	no fixing & following	6 yrs	+	-	+	0.01	0.01	+8	+8	+	macular aplasia	pale optic discs with mild prepapillary fibrosis; vascular attenuation without PPRPE; macular aplasia; several larger areas of sub-retinal white deposits, and confluent nummular intraretinal pigmentation in periphery (34 yrs)

Table 3.2 continued

				Early examination after diagnosis										Most recent examination
Gene mutated	Patient number	Noticed at (months)	First symptom	Age	Night blind	Photo-phobia	Nys-tag-mus	Visual acuity		Refraction (SE)		Kerato-conus	Fundus	Fundus (age)
								RE	LE	RE	LE			
CRB1	2148V1	4	no fixing & following	2 ½ yrs	+	-	+	LP		+9	+8.5	-	nummular RPE alterations temporal macula	full optic discs; normal vessels without PPRPE; macular RPE alterations; several larger areas of nummular intraretinal pigmentation, especially temporal to the macula; diffuse very fine white subretinal deposits (8 yrs)
	3460V1	3	no fixing & following	9 yrs	+	-	+	LP	LP	?	?	+	vascular attenuation; RPE alterations; pale optic disc; maculopathy unknown	not visible due to mature cataract (54 yrs)
GUCY2D	441	3	no fixing & following	3 mo	+	-	+	>LP	>LP	?	?	-	normal	normal (15 mo)
	20955	birth	oculodigital sign	5 yrs	-	-	+	HM (0.5m)	HM (0.5m)	+1.25	+1	+	normal	pink optic disc; narrowing of arterioles; foveal reflex normal; minimal mottling of RPE in the mid- and far periphery (23 yrs)
	21067	6	strabismus	3 yrs	-	+	+	0.05	0.008	+8	+7	-	normal	pale-pink optic disc with drusen; severe narrowing o arterioles; macula is shiny and foveal area shows relative hypopigmentation with pigment alterations; periphery is hypo-pigmented with RPE alterations (24 yrs)
	21557	4	not following light	?	-	-	+	NLP	NLP	?	?	+	normal	?
	22018	2	not following light	2 mo	-	-	+	NLP	NLP	+7	+7	?	grey optic disc	Normal (3 yrs)
	22597	birth	?	?	?	?	+	LP	LP	?	?	+	relatively normal	normal optic discs; attenuated vessels; no pigment alterations of the periphery (25yrs)
RPE65	23207	2	no eye contact	9 mo	+	-	+	LP	LP	+6	+6	-	normal	mild attenuation of retinal vasculature; hypopigmentation of RPE; diffuse RPE alterations (5 yrs)

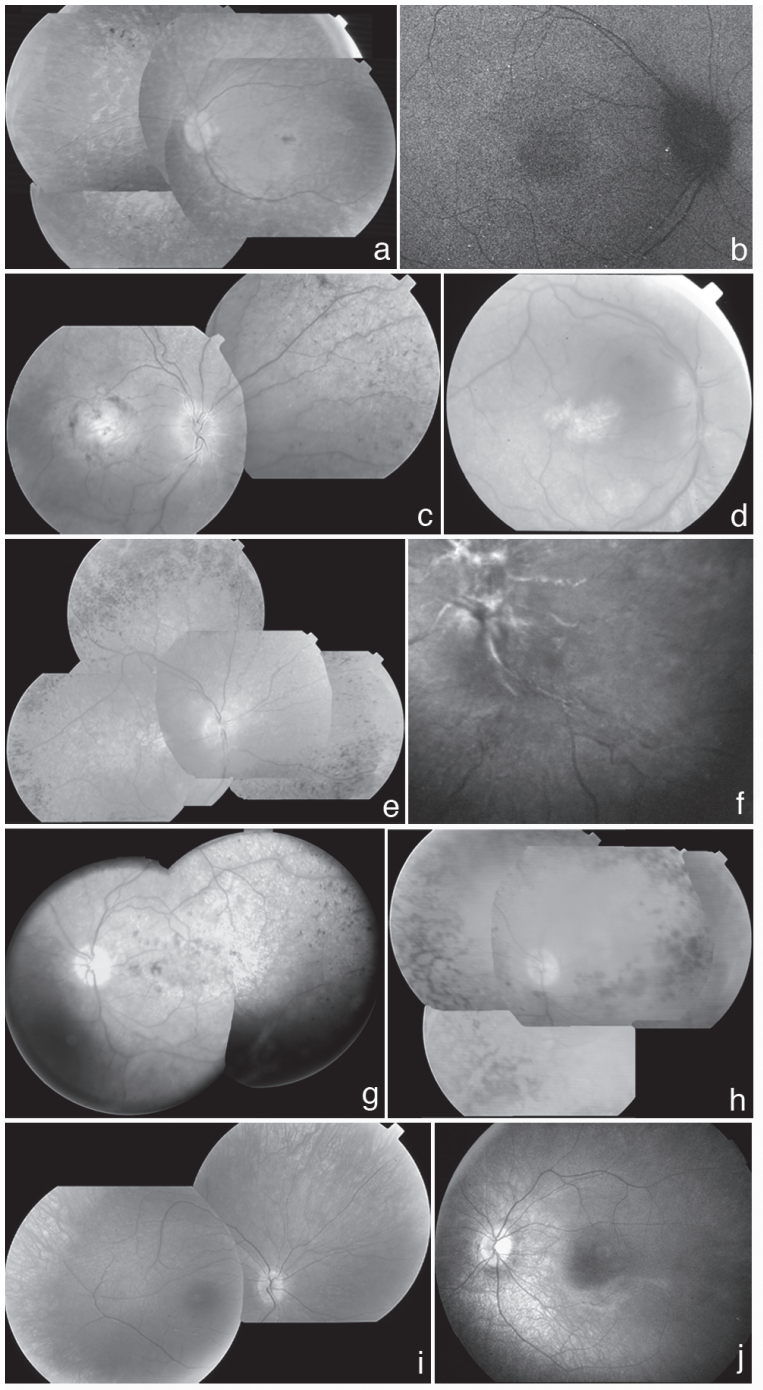
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Figure 3.1 Fundus photographs of patients with LCA

a. Patient 2040V1 (*AIPL1* p.W278X/p.W278W) at age nine: composite of fundus of the left eye (LE), showing macular dystrophy in a bull's eye pattern, diffuse RPE alterations with scarce intraretinal pigment migration with spicular aspect, sub- or deep intraretinal fine white deposits predominantly along vascular arcades.
b. Patient 2040V1 (*AIPL1* p.W278X/p.W278X) at age nine: autofluorescence image of fundus of the right eye (RE), showing fine deposits predominantly along vascular arcades that hyperfluoresce.
c. Patient 21405 (*CRB1* p.C948Y/p.T745M) at age 14: composite of fundus of RE showing an edematous optic disc with perivascular sheating, mild narrowing of the arterioles showing tortuositas with subretinal white dots along arterioles with PPRPE, macular aplasia, atrophic RPE in the periphery with PPRPE, and nummular and a few spicular pigmentations. The posterior pole showed edema with a few intraretinal hemorrhages.
d. Patient 21554 (*CRB1* p.C948Y/c.2842+5G>A): posterior pole of RE at age seven showing an edematous optic disc with preretinal fibrosis, perivascular sheathing, macular dysplasia, and fine, white, deep intra- or subretinal deposits.
e. Patient 21554 (*CRB1* p.C948Y/c.2842+5G>A): composite of fundus of RE at age 12 showing an edematous optic disc with preretinal fibrosis and perivascular fibrotic sheathing without clear PPRPE apart from vessel inferonasal to RE; macular dysplasia remains stable, whereas there is fine nummular intraretinal hyperpigmentation with fine, white, deep intra- or subretinal deposits in the mid- and far periphery.
f. Patient 21554 (*CRB1* p.C948Y/c.2842+5G>A): red free image of the optic disc and inferotemporal peripapillary area of LE at age 12 showing a better visualisation of perivascular fibrotic sheathing.
g. Patient 246V1 (*CRB1* p.K801X/p.Q362X): composite fundus picture of LE at age 14; Note that the optic disc is fairly normal; the macula is dysplastic; limited vascular attenuation with relative PPRPE; nummular hyperpigmentation in the macula and the retinal periphery and multiple white subretinal deposits scattered throughout the fundus.
h. Patient 246V1 (*CRB1* p.K801X/p.Q362X): composite fundus picture of LE at age 30 shows limited visibility due to subcapsular cataract, stable dysplasia of the macula but a dramatic increase in the number of nummular pigmentations in the posterior pole, vascular attenuation; mixed nummular and spicular hyperpigmentation in the retinal periphery and multiple white subretinal deposits scattered throughout the fundus.
i. Patient 21067 (*GUCY2D* p.R768W/p.A946V): Composite of fundus of RE at age 22 showing arteriolar attenuation and mild RPE alterations in the fovea.
j. Patient 23207 (*RPE65* p.R124X/p.F530fs) at age six and a half years: a relatively hypopigmented fundus of LE with mild attenuation of arterioles and diffuse RPE alterations.

CRB1

Genotypes. Eight out of nine patients carrying mutations in *CRB1* were compound heterozygous. The p.C984Y and p.K801X alleles were found in five and four patients, respectively. Two patients with *CRB1* mutations were compound heterozygous for mutations p.C948Y and c.2842+5G>A, and one of them (patient 21551) also carried the p.V96I *AIPL1* sequence variant. The latter sequence change was not considered to be pathologic, because it was identified in heterozygous form in 11 of 186 healthy control subjects. Two new nonsense mutations were identified in *CRB1*: p.Q362X and p.Q1222X.

Phenotypes. Patient 21394 first presented at the age of three months with torticollis presumably related to nystagmus. His fundus was then essentially normal. At the age of five years, his VA had decreased to 0.02 BE. On fundoscopy, he had several characteristics of RP12, including pink optic discs, relatively normal arterioles, and extensive RPE atrophy, both in the posterior pole and in the periphery, with nummular pigmentations. He lacked para-arteriolar preservation of the RPE (PPRPE).

Patient 21405 was first examined in the first year of life because of night blindness. At 0.17 RE and 0.20 LE, her visual acuity was then better than any other patient in this entire study cohort. Funduscopy showed macular and peripheral mild RPE changes as the only abnormalities. At age 14, the typical RP12 phenotype was seen with macular and optic disc edema, a dysplastic macular region, perivascular sheathing, mild arteriolar attenuation, and PPRPE (**figure 3.1c**). Goldmann perimetry performed at the time showed an absolute ring scotoma with relatively well preserved central fields (50º diameter RE, 30º diameter LE).

The first symptom noticed in patient 21551 was a failure to develop eye contact at age four months. When the patient was initially seen at that age, ERG responses were absent. Very limited clinical data were available, but at 13 years of age, he had very poor pupillary reactions with a residual visual acuity and stable hypermetropic refraction. Goldmann visual field testing showed preservation of the central 15º field, albeit with a considerable decrease in sensitivity.

LCA was diagnosed in patient 21554 at three months of age after the oculodigital sign had been noticed and ERG responses were absent. When examined at age three, she preferred a well-lit environment and was reported unable to see in dim light. She had a predominantly horizontal slow nystagmus and esotropia of the right eye. The VA was virtually stable when she was examined at the age of 12. She had by then lost nearly all her ability to recognize basic colors. A small posterior lenticular opacity was present inferior to the visual axis. On fundoscopy she showed the RP12 phenotype and over a nine year period both the aspect and size of the dysplastic regions in both maculae remained virtually stable (**figures 3.1d and 3.1e**).

Perivascular sheathing of retinal vessels around the optic disc was better seen on red free imaging (figure 3.1f).

Patient 21669 was born with palatoschisis. LCA was diagnosed when he was six months of age, when strabismus was noticed and ERG responses were absent. When seen last at age ten years, he showed funduscopic characteristics of RP12 with Coats-like vasculopathy in both eyes. PPRPE was not present.

Patient 246V1 had received a diagnosis at age four months after presenting with nystagmus. Over 30 years, his VA of 0.01 BE and hypermetropia with pronounced astigmatism of +5.0D (-5.5D) 30° RE and +4.5D (-5.25D) 140° LE has remained fairly stable. When last seen at age 30, he preferred bright light. His nystagmus was horizontal. A subcapsular cataract was present in both eyes (LE>RE). Despite the considerable astigmatism, there were no obvious signs of keratoconus. Over a period of 16 years, some of the funduscopically visible RP12 characteristics, such as nummular hyperpigmentation and subretinal deposits, increased (figures 3.1g and 3.1h).

In patient 278V1 the oculodigital sign had been noted since childhood. At the age of 34 years, her VA had decreased slightly due to scarring after acute hydrops of her bilateral keratoconus. Her vision was better in bright light. She had pronounced enophthalmos, esotropia, and nystagmus, which also had a predominant horizontal component. On fundoscopy a steady increase in the white subretinal deposits and nummular pigmentation was noted over the years. The size of the macular aplastic lesions had not changed since birth.

A diagnose of LCA was made in patient 2148V1 at age four months because of a failure to fix and follow objects. ERG responses were absent. At the age of eight years, he preferred bright light and had a VA of only hand movements. He had enophthalmos due to frequent eye–poking, and he displayed horizontal nystagmus. Slit lamp examination findings stayed unremarkable over the years. The nummular intraretinal pigmentation and number of deposits increased. There was some perception of colors as measured with a modified Panel D-15 test.

Patient 3460V1 was a 55-year-old blind woman who was severely mentally retarded presumably due to meningitis in the first weeks of life. When LCA was diagnosed at age three months she was failing to fix and follow objects, and ERG responses were absent. She frequently poked her eyes. After several painful episodes of acute corneal hydrops consequent from keratoconus, her right eye was enucleated at age 53. Because of the presence of a dense cataract, fundoscopy of the left eye was impossible, and, because of mental retardation, visual acuity was unmeasurable.

CRX

In one patient (22654) a heterozygous sequence change (p.Y142C) in the *CRX* gene was identified. Sequencing of the entire gene confirmed the presence of this variant but did not reveal a second change. Segregation analysis of the unaffected consanguineous parents revealed the mother to be homozygous for this sequence change and the father to carry two wild-type alleles. To rule out the possibility that we failed to amplify a wild-type allele by PCR in the mother due to a polymorphic sequence change underneath one of the amplicon primers, we repeated the sequence analysis with different primers and confirmed the homozygosity of the p.Y142C variant in the DNA of the patients' mother. The maternal grandparents had normal vision. The sequence change was therefore considered a benign variant.

GUCY2D

Genotypes. The p.R768W mutation represented eight out of 12 alleles in five of six patients with *GUCY2D*-related LCA. One of the compound heterozygotes carried a novel mutation, p.A946V, which was absent in 93 control individuals.

Phenotypes. LCA was diagnosed in patient 441 at the age of three months after she failed to fix and follow objects. When examined at the age of one year, she frequently poked her eyes. She was able to follow objects, provided she was in a well-lit environment, after having started staring at lights at the age of five months. Her fundus examination had been unremarkable since birth.
Patient 20955 received a diagnosis of LCA in the weeks after birth because of eye poking, which she continued until the age of eight years. ERG responses were absent. At the age of 23 she preferred a dim environment. Her fundus was fairly normal, apart from arteriolar attenuation.

After a diagnosis because of strabismus at 6 months of age, patient 21067 was photophobic as a child, which markedly decreased later in life, and at the age of 22 she instead preferred bright light. She had esotropia of the left eye. Apart from arteriolar attenuation and mild RPE alterations in the fovea, the fundus examination was unremarkable (figure 3.1i).

LCA was diagnosed in patient 21557 at the age of four months, because he failed to fix and follow objects. His ERG responses were absent, and he was completely blind. At the age of 39, he had a divergent strabismus as well as bilateral dense cataracts that precluded fundoscopy.

A diagnosis was made in patient 22018 at the age of two months after he failed to fix and follow objects. He showed the oculodigital sign and episodic head turns. At the age of two and a half years, there was no perception of light, and his pupils were wide and responded only minimally to bright light. Funduscopy was normal.

Patient 22597 belonged to a family with 10 siblings, three of which had LCA. Therefore, an early clinical diagnosis was made soon after birth. There was congenital nystagmus, and her vision had never been better than light perception. Recently, she had acute hydrops of a keratoconus in one eye. At the age of 25 years, attenuation of the retinal vasculature was the only abnormality noted on fundoscopy.

RPE65

Genotype. In this cohort, one patient had LCA due to mutations in *RPE65*. The p.R124X mutation was identified by chip analysis, and a novel frameshift mutation (p.F530fs) was identified by sequence analysis.

Phenotype. Patient 23207 was night blind at the age of six years. He had a very poor VA in the first year of life, with gradual improvement up to 0.16 OD and 0.12 OS, with a refraction of –2.5 OD and –3.5 OS at the age of six years. Nystagmus was present. He had a visual field of 60º and perceived the main colors. His fundus was relatively hypopigmented, with mild attenuation of the arterioles and diffuse RPE alterations (figure 3.1j).

Discussion

The mutation-screening protocol applied in this study consisted of an initial microarray-based mutation analysis, followed by sequence analysis of genes in those patients with LCA in whom single heterozygous mutations were identified. In addition, mutations in patients who were either homozygous or compound heterozygous were confirmed by sequence analysis.

This approach allowed the identification of 37 disease alleles in 19 (33%) of 58 patients with LCA. These represent nine different previously identified and four novel sequence variants. We identified one heterozygous pathologic LCA variant in patient 3460V1 among 58 patients with LCA. Possibly, mutations outside the protein coding exons and their immediate vicinity were missed in this particular patient, as are all heterozygous deletions. Also, the heterozygous variant in 3460V1 could be unrelated to the LCA, since the aggregate heterozygosity frequency of mutations in the *AIPL1, CRB1, CRX, GUCY2D, RPE65,* and *RPGRIP1* genes in the general population is 1.3% assuming an LCA incidence of one in 100,000 (Cremers FPM, unpublished data, 2005).

In 13 families, segregation analysis of the sequence variants was compatible with autosomal recessive inheritance. Of five other families in whom no segregation analysis could be performed, three patients apparently were homozygous for

a disease-causing sequence change. However, the presence of heterozygous deletions cannot be ruled out in these cases.

Mutations in this patient cohort were most frequently identified in *CRB1* (nine cases, 15.5%), followed by *GUCY2D* (six cases, 10.3%), *AIPL1* (three cases, 5.2%), and *RPE65* (one case, 1.7%). No pathologic mutations were found in *CRX*, *RDH12* (in 22 patients), and *RPGRIP1.* The infrequent involvement of *CRX, RDH12,* and *RPGRIP1* in LCA is in accordance with the results of previous studies[6,8,27,30]. In addition, it is noteworthy that *RPE65* seems to account for only a minority of cases (1.7%) in comparison to reported frequencies in other studies of 3% to15%[31-34]. This variation may be due to the relatively small patient cohort, the variable mutation detection strategies and the variable inclusion criteria applied by different groups. In general, mutations in the *RPE65* gene are more frequently observed in patients with early-onset RP rather than LCA[35] (Yzer S, unpublished results 2005).

All three patients carrying *AIPL1* mutations were homozygous for the p.W278X allele. Previously, nine patients from different ethnic backgrounds were described to be homozygous for this change by Dharmaraj *et al*[36]. The phenotype of these patients consisted of severe night blindness, some degree of maculopathy, attenuated vessels, and spicular intraretinal pigment migration in the periphery after several years of evolution. Keratoconus was observed in six of 19 patients with distinct *AIPL1* mutations. The patients described in this study showed an apparently normal fundus on initial examination, which progressively evolved into peripheral spicular intraretinal pigment migration in early childhood. High hyperopia and a very poor visual acuity appear to be consistent findings. Night blindness is a frequent, albeit variable, characteristic[6,36].

Eight of nine patients with *CRB1* mutations showed either all or some funduscopic characteristics of the RP12 phenotype, including relatively normal optic discs and vessels, a distinct maculopathy and a nummular rather than a spicular type of pigmentation. Yellow-white retinal dots were present in eight patients and PPRPE in three. Findings less frequently observed in RP12 or patients with *CRB1* mutations, such as Coats-like exudative vasculopathy, vascular sheathing, optic disc edema, maculae dysplasia, and keratoconus were also observed in our patients. Moderate to high hyperopia and nystagmus are also associated with *CRB1*-associated LCA[4,6,8,37]. This RP12 phenotype is easily distinguishable from other RP-like phenotypes.

The *CRX* p.Y142C variant has been identified in heterozygous form in a patient with autosomal dominant LCA (Kaplan J, personal communication, 2004). In our cohort one patient from a consanguineous marriage was a heterozygous carrier of this variant. Sequence analysis revealed the healthy mother to be homozygous for this sequence change, suggesting that the p.Y142C change is nonpathogenic. Because the maternal grandparents had normal vision, a metabolic interference model

in which homozygous mutations abolish each other's pathologic effects, seems unlikely. Therefore, we conclude that 33 of 116 disease alleles were identified by chip analysis alone.
Mutations in the *GUCY2D* gene in LCA were first reported in Mediterranean families[10]. In the LCA cohort of this study, the p.R768W mutation was found in eight (67%) of 12 alleles. Four of the five patients carrying this allele were of Belgian or Dutch origin, and the fifth was of Belgian/Moroccan descent. Segregation analysis showed that the p.R768W allele came from her Belgian mother. Hanein *et al*[6] found the p.R768W allele in only 1 of 75 *GUCY2D* alleles in 38 patients, most of which were from Mediterranean countries. This difference in frequency of the p.R768W mutation may be suggestive of a founder effect in the northwestern region of Europe. The three patients with the homozygous p.R768W *GUCY2D* mutation are severely affected, did not show light perception at initial examination, and had no signs of improvement later in life. In most patients, the initial fundoscopy was normal which, later in life, showed attenuated vasculature and subtle pigmentations. In accordance with previous findings by Dharmaraj *et al*[30], refraction in *GUCY2D*-related patients with LCA varies from mild to severe hyperopia with VA showing a spectrum from no light perception to 0,1. In contrast, Hanein *et al*[6] generally found hypermetropia (> +7).

A novel frameshift mutation, p.F530fs, was identified in the *RPE65* gene in a patient heterozygous for p.R124X. The phenotype of this patient is similar to previously published phenotypes with very poor visual acuity early in life that gradually improves, myopia, and some residual visual field function. Overall, the phenotype of patients with LCA with *RPE65* mutations seems relatively mild in comparison to other forms of LCA[6,34,35].

Our patient cohort was relatively small, but our clinical data suggest that patients with homozygous p.W278X *AIPL1* mutations have a very poor visual acuity, with hyperopia and an initially normal fundus, which progresses to an RP-like fundus in early childhood. In general, *GUCY2D*-related LCA results in the most severe visual impairment with a relatively normal fundus in the mid-20s. Furthermore, this study supports the existence of a fairly typical and consistent phenotype of *CRB1*-associated LCA, including a maculopathy ranging from macular RPE alterations to macular aplasia, fine white sub- or deep intraretinal drusen-like deposits, and nummular rather than spicular hyperpigmentation in both maculae and periphery. The phenotypical data also confirm that PPRPE is a rather inconsistent feature in patients with *CRB1* mutations, probably due to its transient nature.

In this study, *CRB1* was the most frequently involved gene (9/19 patients) in a cohort of predominantly Belgian and Dutch patients. In a Spanish LCA cohort, mutations in *CRB1* turned out to underlie two thirds of all cases with mutations in the known LCA genes[38]. In contrast, no *CRB1* mutations were detected in a Canadian cohort of 24 patients with LCA, suggestive of sizeable geographic differences in the occurrence of mutations[39].

Taken together, the clinical findings in our patients suggest that both photophobia and night blindness do not seem to be reliable clinical features that can be used to direct gene analysis, as was proposed by Hanein *et al*[6]. Indeed, within the *AIPL1*-, *GUCY2D-,* and *CRB1*-associated patient cohorts in our study, both photophobia and night blindness were noted.

A comprehensive mutation analysis study of all currently known LCA genes has previously been performed[6]. Excluding this study's findings in the *TULP1* gene, as the *TULP1* gene was not analyzed in our study, mutations were identified in 44.4% of the disease alleles using a combination of linkage analysis of known genes, denaturing high-pressure liquid chromatography mutation scanning, and direct sequencing. Our approach (i.e., a first-pass mutation detection of known mutations with the LCA microarray chip followed by sequence analysis of patients with heterozygous mutations) allowed the detection of 31.9% (37/116) of disease alleles.

Hence, about 72% (31.9/44.4) of the expected disease alleles were identified with a relatively small effort. We therefore believe that the LCA mutation chip offers a cheap and efficient first-pass screening test. This robust technique is generally applicable and allows more carefully directed efforts of subsequent standard sequence analysis. Furthermore it is independent of a detailed clinical history necessary for a decisional molecular diagnostic flowchart, as outlined by Hanein *et al*[6]. Finally, by regular addition of newly identified alleles, the efficiency of the microarray will continu to improve.

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4

A p.Y368H *RPE65* founder mutation is associated with variable expression and progression of early-onset retinal dystrophy in 10 families of a genetically isolated population

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Abstract

We investigated the ophthalmologic characteristics and molecular cause of a retinal dystrophy in 14 patients belonging to ten families from a multigenerational pedigree in a genetic isolate in the Netherlands. All affected individuals experienced night blindness from early childhood and most of the patients had nystagmus. Visual acuity loss in these patients at first examination varied considerably (ability to observe hand movements at 2 m to 20/50). Moreover, visual acuity either improved (four patients), deteriorated (four cases) or was stable over a mean nine year follow-up period. Rod electroretinogram (ERG) responses were unrecordable in all patients, whereas cone responses (ERG) were recordable in early childhood in five patients. Peripheral vision was relatively well preserved. Based on previously published phenotypes associated with *RPE65* mutations we hypothesized the involvement of the *RPE65* gene. We found homozygosity-by-descent of a D1S2803 marker allele located very near the *RPE65* gene. In nine families patients were homozygous for a *RPE65* p.Y368H mutation; in one family a patient was compound heterozygous for the *RPE65* p.Y368H and c.IVS1+5G>A mutations. Based on the estimated total number of patients with congenital retinal dystrophy in the genetic isolate and the DNA analysis of healthy controls of this population we estimate a carrier frequency of ~1 in 20 individuals rendering a significant risk of blindness in offspring of patients, their heterozygous siblings and other heterozygous individuals of the genetic isolate.

Introduction

Autosomal recessive retinal dystrophies cause visual impairment in approximately 1 in 4000 individuals worldwide[1]. The non-syndromic forms are highly heterogeneous and can be classified into clinical subgroups, the most frequent ones being retinitis pigmentosa (RP), cone- and cone-rod dystrophies, and Leber congenital amaurosis (LCA). LCA represents the most severe phenotype with an onset of symptoms before the age of six months, visual acuity below 20/400, a searching nystagmus, sluggish pupillary reactions and a non-detectable ERG. Visual fields are usually not measurable[1]. Photophobia is only occasionally reported in LCA[2]. Patients with juvenile and early-onset RP present with night blindness in early childhood, usually before the age of two years. They do not show searching nystagmus[3] and have a relatively well preserved macular function. Central vision is often lost in the second or third decade of life[4].

The cloning of more than 20 genes allows the molecular characterization of approximately 50% of the autosomal recessive inherited retinal dystrophy cases (www.sph.uth.tmc.edu/Retnet[5,6]). To establish a useful clinical prognosis for patients a well defined genotype-phenotype correlation is required.

Although the general population in the Netherlands is relatively outbred, there are a few examples of autosomal recessive diseases caused by Dutch founder mutations. Batten disease was found to be due to identity-by-descent in a highly inbred family[7]. A frequent LDL receptor mutation originating from Dutch 17th century settlers causes familial hypercholesterolaemia in South Africa and Canada[8]. The RP12 locus was mapped through linkage analysis in a genetic isolate from the northwest of the Netherlands[9], which was followed by the cloning of the underlying gene, *CRB1*[10].

In 1959 Schappert-Kimmijser *et al* described an isolated Dutch population living on a former island with a relatively high frequency of LCA[11]. In 1637 this community suffered from the plague, which killed half the population. After the island was connected to the mainland in 1941, the 4000 residents could be regarded as descendants of at most 150 ancestors. Since then, the population size has quadrupled, mostly through endogamous marriages.

We re-evaluated this population and established a new multigenerational pedigree, consisting of ten nuclear families with congenital retinal dystrophy. We undertook detailed clinical and molecular studies and identified two *RPE65* mutations, one of which represents a frequent founder mutation in the genetic isolate.

Patients and methods

Fourteen patients with congenital retinal dystrophy, 25 of their non-affected siblings, and 19 parents were asked to participate in this study. The study protocol adhered to the regulations of the Declaration of Helsinki.

Clinical analysis

Parents and patients were questioned about pregnancy, delivery, the age of onset of symptoms, night blindness, photophobia and birth defects. Ophthalmic examinations were done by one of us (JS, FNB, MMvG). All children were seen between 1978 and the present. The mean follow-up period was nine years and ranged from two to 15 years.

Ophthalmic examinations included best corrected visual acuity with age adapted functional tests, such as Teller acuity cards in the preverbal period and the Lea Hyvärinen test for children who were able to match or name pictures. From the age of six years Snellen charts were used. Objective refractive error was measured after cycloplegia. Pupillary reactions were tested and nystagmus and photophobia were evaluated. Night blindness was tested by observing the patients' behavior in a dim lighted room. The anterior segment was examined using a slit lamp, followed by dilated fundoscopy. Kinetic visual fields were measured with Tübinger perimetry (with a ten apostilbs background) in13 patients. Electroretinographic recordings were made in all patients but one, and were done at two university hospitals. Color vision was tested with the Ishihara test for color blindness, Hardy Rand Rittler (H-R-R) charts, and Lanthony's saturated D-15 test. Dark adaptometry was obtained in three patients with the Goldmann-Weekers dark adaptometer. Fundus photographs were taken with a Topcon retinal camera, model TRC-F.

Molecular analysis

Venous blood was collected and DNA was extracted using a previously described protocol[12]. DNA was analyzed using the highly polymorphic DNA marker D1S2803 (AFMB361YD5) located 2.5 kilobases (kb) proximal to the *RPE65* gene and D1S2895 (AFMA099WC1), located approximately 2.7 megabases (Mb) proximal to the *RPE65* gene according to the human genome project working draft at http://genome.cse.ucsc.edu (freeze June 2002).

Amplification reactions were done with 50 ng genomic DNA, 6 pmol of each primer, 200 µM of dATP, dTTP, dGTP, and dCTP, 2 pmol $\alpha[^{32}P]$-dCTP. 1 × SuperTaq buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.1% (wt/vol) Triton X-100; 0.01% (wt/vol) gelatine), and 0.1 U Taq DNA polymerase (Invitrogen). Cycling parameters consisted of 94°C for 4 min, followed by 30 cycles of 92°C for 1 min, 55°C for 2 min, 72°C for 1 min, and a final extension of 6 min at 72°C. Polymerase

chain reaction (PCR) products were separated on a 6.6% denaturing polyacrylamide gel (acrylamide:N,N'-methylene-bisacrylamide 19:1), containing 100 mM Tris-borate, 2 mM EDTA pH 8.3, and 8.3 M Urea. After electrophoresis and drying of the gels the bands were visualized by overnight exposure on an X-Omat AR film.

After strong evidence of involvement of the *RPE65* gene was obtained, we analyzed the nucleotide sequence of the 14 exons of the *RPE65* gene in genomic DNA of one healthy individual and three probands (BII-5, GII-1, and HII-1). We slightly adapted the PCR conditions described by Marlhens and coworkers[13]. The PCR buffer contained 3 mM $MgCl_2$ and we employed the following PCR cycling temperatures: 30 s at 94°C, 30 s at 56°C (exons 4 to 10) or 60°C (exons 1 to 3 and 11 to 14), and 1 min at 72°C (30 cycles). Cycle sequencing was done by using the same gene specific PCR primers[13] to amplify the 14 exons and flanking introns sequences of the *RPE65* gene. The PCR products were purified by using the Millipore purification system and analysed on the ABI3730 or ABI3100 DNA analysers. Automatic analysis was done by ABI basecaller.

The presence of the c.1156T>C (p.Y368H) founder mutation was tested in 96 healthy controls from the genetic isolate after isolating DNA from buccal swabs. Mucous membrane cells were collected by firmly brushing the inside of the cheek, after which 600 µl 50 mM NaOH were added and the samples were heated for 5 min at 95°C. Buccal swabs were removed and 60 µl Tris pH 8.0 was added. After centrifugation at 13 000 rpm for 10 min, the supernatant was collected and used for PCR of exon 10 of the *RPE65* gene and subsequent restriction fragment analysis using *Nla*III.

Eventually, the same analysis was used to test a panel of 94 healthy controls from the Netherlands, 75 Dutch patients with autosomal recessive or isolated retinitis pigmentosa, and 86 patients with LCA (15 patients from the Netherlands, 18 from Germany, 2 patients from the USA, and 51 from Canada).

Results

Clinical evaluation

The relevant clinical data are summarised in table 4.1. None of the 14 newborns followed objects or made eye contact, leading their parents to suspect visual impairment within the first three months of life. Most of the children were seen by an ophthalmologist within their first year. All experienced night blindness. Nine children went to regular schools while the others attended schools for the visually impaired. Two of the children (AII-2, HII-5) were considered to have psychomotor retardation with an autistic-like contact disorder. One patient (BII-5) underwent surgery because of polydactyly.

A wide range of visual acuities was observed at the first examination. On follow-up visual acuity varied: in nine patients (AII-2, AII-3, BII-5, CII-2, DII-1, EII-1, FII-2, GII-1 JII-2) it remained relatively stable or seemed to improve, whereas in four (HII-1, HII-2, HII-5, KII-6) it deteriorated (table 4.1). All but two children showed early-onset nystagmus or developed nystagmus within the first six years of life. Eight patients showed a mild torticollis. None of the patients had photophobia; on the contrary, all children adored bright light. Two patients had moderate hypermetropia, one of whom (BII-5) had a father and an older sister who were also hypermetropic but did not have a retinal disorder. The other hypermetropic individual (HII-5) had two affected siblings who were near emmetropic. Three patients had moderate myopia (DII-1, JII-2, KII-6). Six children had strabismus, which led to an amblyopic eye in two (table 4.1). Pupillary reactions were normal and symmetrical in all. None of the patients had cataract.

Early fundoscopy was either normal or showed relative hypopigmentation or subtle changes in the retinal pigment epithelium. Macular reflexes were still present in all patients, while foveal reflexes were present in half the affected individuals. At the most recent examination retinal vessels were moderately or severely attenuated and the optic discs were pale in all patients. In five patients the periphery appeared hypopigmented. All patients had subtle subretinal pigmentary changes. No intra-retinal bone-spicules were seen. Macular reflexes were still present later in life, whereas the foveal reflexes disappeared in all but one patient (table 4.1 and figure 4.1).

Seven of the 14 patients were unable to perform color vision tests. In the other seven patients color vision was severely disturbed and had an overall tendency to the tritan axe (blue-yellow axe). Tübinger visual field assessment was done in 13 patients. Though visual field defects were slightly progressive over the years, the peripheral fields remained relatively stable in ten patients. In only three was there marked deterioration. Dark adaptation tests were done in three patients and showed monophasic curves with a markedly increased rod and cone segment. ERGs were done within the first three years of life in 12 patients. In seven patients no responses were detected. In four patients, significantly reduced photopic responses were measured, with absent scotopic responses. In only one patient (HII-5) were both scotopic and photopic responses recorded (table 4.1).

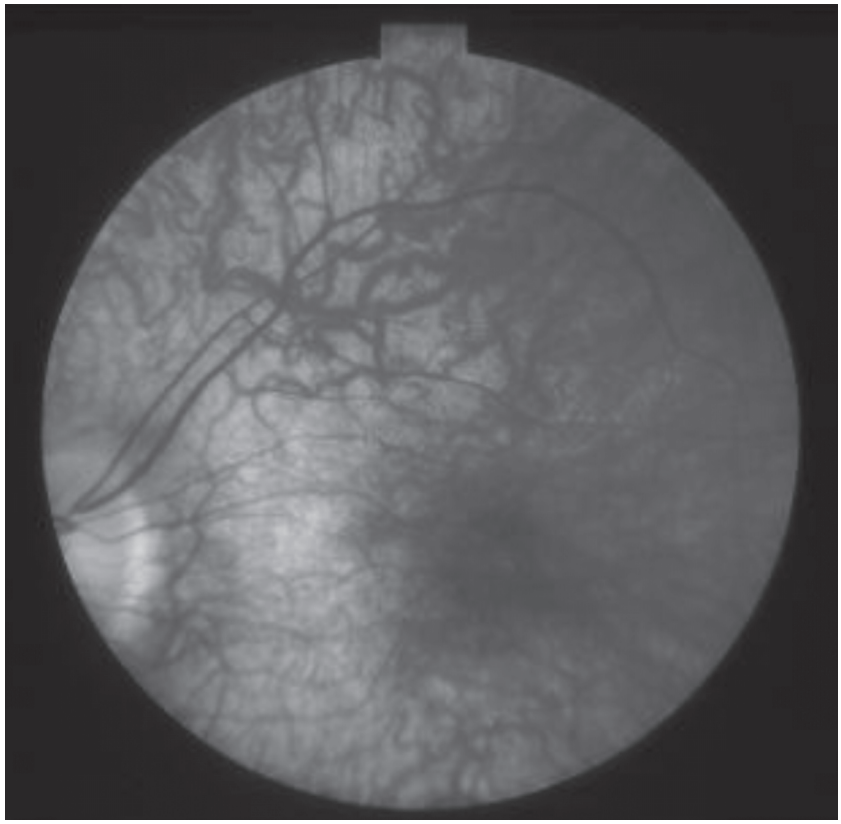

Figure 4.1 Fundus photograph of patient HII-1 at the age of eight years with mutations in the *RPE65* gene
Pale optic disc, moderately attenuated vessels, hypopigmented fundus with a relatively spared maculae.

Genealogy

Genealogical studies showed that all ten families were related through at least one and in most cases several common ancestors (figure 4.2). Some patients were first degree cousins (for example, patients from families B and C; patients from families D and E); others were only connected through a common ancestor living in the 18th century eight generations ago (for example, families F and G). We therefore reasoned that the retinal dystrophy in the ten families most probably caused by homozygosity-by-descent of one or a small number of autosomal recessive mutations.

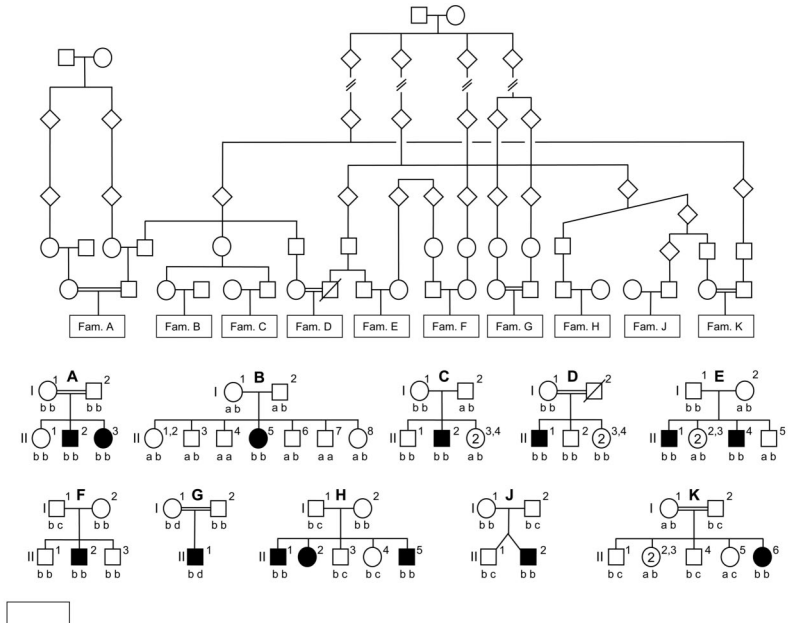

Figure 4.2 Anonymous and simplified pedigree structure of part of the genetic isolate (top) and D1S2803 marker alleles in the families with early-onset retinal dystrophy (bottom)

The individuals in the oldest generation were born in the second half of the 18th century. Double bars reflect known consanguinity. Numbers in the circles in the youngest generation indicate the number of individuals that share the same alleles for marker D1S2803. All affected individuals except GII-1 are homozygous for allele b, suggesting the presence of a founder mutation in the affected individuals through homozygosity-by-descent. Patient GII-1 is heterozygous for marker D1S2803, suggesting the presence of two different *RPE65* mutations. ◇, unaffected male or female; □, male; ○, female; black figures, affected persons.

Table 4.1 Ophthalmologic data for 13 patients with a homozygous p.Y368H *RPE65* mutation, and one patient (GII-1) with compound heterozygous *RPE65* mutations
*amblyopia, ERG (electroretinogram), FR (fovea reflex), HM (hand movements), MR (macula reflex), OD (right eye), OS (left eye), OU (both eyes), PR (photopic responses), RPE (retinal pigment epithelium). SR (scotopic responses).

Individual	First Visual Acuity (age)		First Fundoscopy	Recent Visual Acuity (age)		Recent Fundoscopy	Objective Refraction Spherical Equivalent		ERG (age)
	OD	OS		OD	OS				
AII-2	OU: 20/60 (4y)		normal	20/800(15y)*	20/60	severely attenuated vessels, MR:+, peripheral pigmentary alterations	-0,25	-0,25	SR and PR: non-recordable (1y)
AII-3	20/60 (9y)	20/100	normal	20/50 (17y)	20/80	optic disc pallor, moderately attenuated vessels, MR:+, peripheral pigment alterations	plano	-1,00	SR: non-recordable and PR: significantly reduced (2y)
BII-5	20/200 (4y)	20/200	normal	20/200 (15y)	20/125	optic disc pallor, moderately attenuated vessels, macular pigmentation, midperipheral pigment alterations	+2,75	+3,50	
CII-2	20/250 (3y)	20/200	subtle RPE changes, MR:+, FR:+	20/150 (9y)	20/100	optic disc pallor, moderately attenuated vessels, MR:+, FR:+, mottled pigmentation in periphery	plano	plano	SR and PR: non-recordable (3y)
DII-1	HM 2m (6y)	HM 2m	hypopigmentated fundus, MR:+, FR:-	20/400 (21y)	20/400	optic disc pallor, attenuated vessels, hypopigmentated fundus with peripheral pigmentations	-3,50	-2,50	SR and PR: non-recordable (3y)
EII-1	20/60 (6y)	20/80	normal	20/100 (8y)	20/80	moderately attenuated vessels, MR:+, RPE atrophy	-0,50	plano	SR and PR: non-recordable (1y)
EII-4									SR and PR: non-recordable (1y)
FII-2	20/300 (1y)	20/200	subtle RPE changes	20/200 (8y)	20/200	optic disc pallor, attenuated vessels, irregular pigment alterations	plano	plano	
GII-1	OU: 20/200 (1y)		hypopigmentated fundus, MR:+, FR:-	OU: 20/200 (2y)		optic disc pallor, mildly attenuated vessels, MR:+, hypopigmentated midperiphery	plano	plano	SR and PR: non-recordable (1y)
HII-1	20/50 (4y)	20/150	subtle RPE changes, MR:+, FR:+	20/60 (19y)	20/200	optic disc pallor, mildly attenuated vessels, MR:+, hypo-pigmentated fundus, mottled pigmentation in periphery	+0,50	+0,50	SR: non-recordable and PR: significantly reduced (4y)
HII-2	20/300 (4y)	20/300	subtle RPE changes, MR:+, FR:-	20/600 (19y)	HM 1m*	optic disc pallor, mildly attenuated vessels, MR:+, hypo-pigmentated fundus, mottled pigmentation in periphery	+0,50	+0,50	SR: non-recordable and PR: significantly reduced (2y)
HII-5	20/150 (2y)	20/150	subtle RPE changes, MR:+, FR:-	20/250 (10y)	20/300	optic disc pallor, moderately attenuated vessels, MR:+, mottled pigmentation in periphery	+4,25	+4,25	SR and PR: significantly reduced (1y)
JII-2	20/100 (4y)	20/100	hypopigmentated fundus, MR:+, FR:-	20/50 (12y)	20/40	moderately attenuated vessels, MR:+, midperipheral pigmentary changes	-4,50	-4,50	SR: non-recordable and PR: present (1y)
KII-6	20/60 (6y)	20/80	subtle RPE changes, MR:+, FR:-	20/150 (10y)	20/150	moderately attenuated vessels, MR:+, hypopigmentated periphery, RPE granulated, some peripheral pigmentations	-1,00	-2,50	SR and PR: non-recordable (0.5y)

Molecular genetic analysis

The phenotypes described above suggested the involvement of the *RPE65* gene, as previous studies have implicated this gene in patients with LCA and early-onset severe rod-cone dystrophy quite similar to the clinical presentation described in patients in this study[6,14,15]. To test this hypothesis, we undertook linkage analysis employing the DNA marker D1S2803 located only 2.5 kb proximal of the *RPE65* gene. Eight of 10 families were partially or fully informative (figure 4.1). All affected individuals except patient GII-1 are homozygous for D1S2803 allele b. Given that allele b has a frequency of 0.32 (www.gdb.org) the likelihood that it occurs in 19 of 20 alleles from the probands of the nuclear families is $(0.32)^{19} \times 0.68 = 8.6^{-11}$. This calculation strongly suggests the presence of a homozygous founder mutation in these patients. Moreover, analysis of marker D1S2895, situated three cM proximal to the *RPE65* gene, also revealed homozygosity of one allele in five of eight informative families (data not shown).

Subsequently, we analysed the nucleotide sequence of the 14 protein coding exons of the *RPE65* gene in three probands (BII-5, HII-1, and GII-1) and one control DNA sample. We identified a homozygous c.1156T>C nucleotide change (p.Y368H) in patients BII-5 and HII-1. Patient GII-1 was found to be compound heterozygous for the c.1156T>C nucleotide change and a splice site mutation in intron 1 (IVS1+5G>A) (data not shown).

We analyzed the segregation of the exon 10 mutation by RFLP analysis as the c.1156T>C change introduces a *Nla*III restriction site. The normal PCR product of exon 10 contains one *Nla*III site resulting in restriction fragments of 41 and 205 bp; a mutant allele is cut into 41, 85, and 120 bps. As illustrated for family B, patient BII-5 was indeed homozygous for the mutant restriction pattern (figure 4.3). Five healthy siblings were heterozygous for this mutation and two unaffected siblings did not carry a mutant allele. *Nla*III RFLP analysis of the ten families showed that all patients except GII-1 were homozygous for the c.1156T>C nucleotide change and that all parents except GI-1, were heterozygous carriers of this mutation. GI-1 instead carried the IVS1+5G>A mutation. Among 25 unaffected siblings tested, 17 were heterozygous for the p.Y368H mutation and eight did not carry this mutation. No other mutations or polymorphisms were found in the three patients and one control DNA sample analyzed.

Using the *Nla*III RFLP analysis three of 96 healthy controls from the genetic isolate were shown to carry the p.Y368H founder mutation heterozygously. We also investigated the frequency of this founder mutation in a panel of 86 patients with LCA from different Caucasian populations, 75 patients with autosomal recessive or isolated retinitis pigmentosa from the Netherlands, and 94 healthy controls from the Netherlands. The p.Y368H mutation was not found in the random control group or

in the LCA cohort but was found heterozygously in one Dutch patient with retinitis pigmentosa and early-onset visual loss.

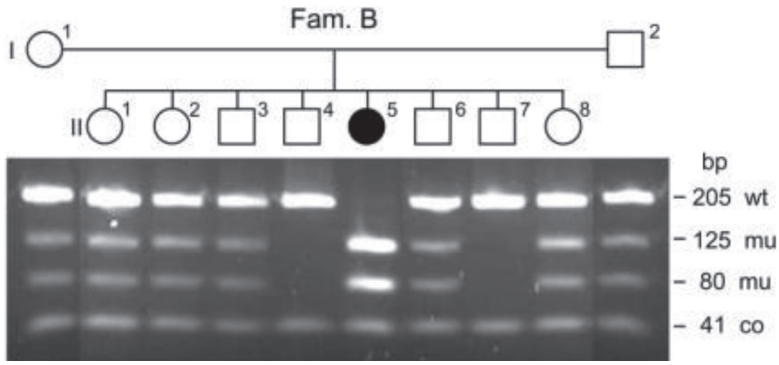

Figure 4.3 Restriction fragment length polymorphism based co-segregation analysis of *RPE65* p.Y368H mutation in family B of the genetic isolate
NlaIII cuts the 246-bp PCR product in fragments of 41 and 205 bp in wildtype alleles and in 41, 80, and 125 bp in mutant alleles. Five non-affected siblings are heterozygous carriers of the p.Y368H mutation; two carry wild-type alleles. Wt, wild-type; mu, mutant, co, constant. Siblings are not depicted in order of age to preserve anonymity.

Discussion

We found 14 patients with a congenital retinal dystrophy belonging to ten related families from a former genetic isolate in the Netherlands. The age of onset of the retinal dystrophy suggests LCA, but several characteristics of the disease – for example, visual acuity, visual fields and night blindness - strongly support the diagnosis of early-onset retinitis pigmentosa. We therefore classify the phenotype of our patients as an early-onset severe retinal dystrophy. Based on a comparison of the phenotypes observed in patients of our pedigree with previous genotype-phenotype studies[6,14,15], we hypothesized that the *RPE65* gene represented a good candidate gene. Linkage analysis of a highly polymorphic marker located very near the *RPE65* gene revealed homozygosity-by-descent in nine of ten families. We found homozygosity for a p.Y368H mutation in nine families, and compound heterozygosity of the p.Y368H and IVS1+5G>A mutations in one patient of the other family. The p.Y368H mutation has most probably been inherited from a common ancestor of all 10 families born in the 18th century or before. The IVS1+5G>A mutation is the most frequently encountered LCA associated *RPE65* mutation (ref. 5 and references therein).

Both *RPE65* variants described above have been found previously in patients with similar phenotypes and were not found in 50 random control individuals from the general population. Moreover, the Y368 residue is conserved among human, bovine, canine, rat, chicken, and salamander[17].

Our results clearly show that a homozygous p.Y368H mutation in the *RPE65* gene can be associated with a large variability in visual acuity. Moreover, over a mean nine year follow up period, visual acuity either remained stable, seemed to improve slightly, or deteriorated. The observed improvement can be explained by visual and mental development that may influence results of visual acuity testing favourably. Within families, concordance of progression of the disease was observed. At present, six patients are over 20 years of age and have stable visual acuity. Based on our findings it is not possible to give patients with a homozygous p.Y368H mutation an accurate prediction on their future visual acuity. An explanation for the variable clinical outcome cannot be found in the different functional consequences of the various mutations. Clearly, other genetic or environmental factors influence visual acuity and its progression. No other patients have been described with a homozygous *RPE65* p.Y368H mutation. Patients who are compound heterozygous for this mutation and a p.R91W mutation showed a more severe disease course[16,18].

In a previously identified patient with the same compound heterozygous mutations as patient GII-1, the age of onset was five years, with a visual acuity of 20/100 (both eyes) and three diopters of hypermetropic correction[18], whereas our patient was visually impaired since birth, had a visual acuity of 20/200, and was emmetropic by the age of three years. Again, knowledge of the mutations seems of no predictive value for disease course.

On initial examination patients had normal fundi or mild changes, whereas eventually all fundi showed severe and more prominent changes, similar to the development as described by Lorenz and co-workers[16].

Four patients described by Lorenz *et al* and two described by Felius *et al* were hypermetropic, as were nine of 12 patients with *RPE65* mutations described by Lotery *et al*[16,18,19]. Within our group of 13 patients with a homozygous p.Y368H mutation only two were hypermetropic. One hypermetropic patient had two unaffected relatives who were hypermetropic, suggesting that other genetic factors were involved. The other patient had two affected siblings who were near emmetropic. Thus the homozygous p.Y368H mutation, in contrast to other combinations of *RPE65* mutations, is not associated with hypermetropia.

In a study of the same population 43 years ago, Schappert-Kimmijser and coworkers ascertained 13 LCA patients in eight families[11]. It can be predicted that most if not all of these patients carried the p.Y368H founder mutation. Assuming that half of the patients seen by Schappert-Kimmijser and co-workers are still alive

and since we have not investigated this population in depth, a conservative estimate would be that there are 25 patients with early-onset severe retinal dystrophy in the current population (consisting of 16.500 people) carrying the p.Y368H founder mutation homozygously. Assuming heterozygosity of this mutation in 1/660 individuals, we deduced a carrier frequency of ~1 in 13. Analysis of 96 healthy individuals from the genetic isolate yielded a p.Y368H carrier frequency of 3.1% (95% confidence interval, -0.4% - 6.6%). Based on the observed carrier frequency of the p.Y368H founder mutation in this former genetic isolate, patients and heterozygous carriers with partners from this population have a risk of ~1 in 56 and ~1 in 112, respectively, that anyone of their children will develop early-onset severe retinal dystrophy.

RPE65 has a crucial, but as yet not fully understood, role in the isomerisation of all-*trans*-retinal to 11-*cis*-retinol in the retinal pigment epithelium[20,21]. In *RPE65* deficient mice and dogs, pharmacological and somatic gene therapy based treatments, respectively, were successful[22,23]. It is clear that further assessments of long term safety and efficacy are required for various treatment strategies and, as treatment may need to be applied very early in life, ethical aspects will have to be considered. Nevertheless, patients carrying defects in the *RPE65* gene might become eligible for future clinical trials, underscoring the importance of their early clinical and molecular genetic identification.

Acknowledgements

We thank the patients, their family members and their general practitioners for their kind cooperation, Dr E. Hennekam for genealogical studies and M.F.W.J. Ariaans, M. Zonneveld, and Dr R. Roepman (University Medical Center Nijmegen) for their assistance in several aspects of these studies. This study was supported by the 'Stichting Wetenschappelijk Onderzoek het Oogziekenhuis prof. dr H.J. Flieringa' Rotterdam (LIvdB, FPMC), Retina Netherlands (LIvdB, FPMC), and the Canadian Foundation Fighting Blindness (Toronto, Ontario; RKK, FPMC).

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5

CRB1 heterozygotes with regional retinal dysfunction: implications for genetic testing of Leber congenital amaurosis

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Abstract

Objectives

To test humans with heterozygous *CRB1* mutations for possible clinical or functional retinal changes and evaluate whether an LCA patient with *CRB1* mutations, not consistent with previously described *CRB1* phenotypes, carried a modifier allele in another LCA gene.

Methods

Seven unrelated heterozygous carriers of *CRB1* mutations, who's children have LCA, underwent phenotyping by full eye examinations (indirect ophthalmoscopy and slit lamp biomicroscopy) and functional testing (standard full field electroretinography and multifocal ERG). For genotyping of the LCA patients and their parents, we performed dHPLC analysis followed by sequence analysis of *CRB1*, followed by sequence analysis of the *AIPL1* and *CRX* genes to identify a putative modifier effect in a patient with an atypical *CRB1* phenotype.

Results

We found reduced full-field ERG b-wave amplitudes with scotopic –2dB flash (140 μV, $p<0.05$), normal full field cone ERGs, and significant regional retinal dysfunction on mfERG in five out of seven carriers of *CRB1* mutations. We identified a known *AIPL1* mutation (p.R302L) as being a potential modifier allele in a LCA patient carrying two *CRB1* mutations, with a prominent maculopathy.

Conclusions

In human heterozygotes of *CRB1* mutations, we found distinctive regional retinal dysfunctions by multifocal ERG measurements consistent with the focal histological abnormalities reported for the two *CRB1* knockout mice models. This phenotypic finding may identify *CRB1* carriers and point to the causal gene defect in affected LCA offspring, significantly facilitating the molecular diagnostic process. We also provide evidence suggestive of a modifier allele in *AIPL1* in a case of LCA with prominent atrophic macular lesions and homozygous defects in *CRB1*.

Introduction

Leber congenital amaurosis (LCA, MIM 204000) is a severe, currently untreatable, congenital retinal dystrophy that leads to blindness. Theodor Leber defined LCA in 1869 as a congenital form of retinitis pigmentosa (RP) with profound visual loss at or near birth, wandering nystagmus, amaurotic pupils, a pigmentary retinopathy and autosomal recessive inheritance[1]. A severely reduced electroretinogram (ERG) was later added to this definition as this distinguishes it from a complex set of other overlapping congenital retinal disorders[2]. LCA creates an appreciable burden on the affected child, the family and society, as the blindness is lifelong and commences at birth. It has a worldwide prevalence of 1 in 100,000 newborns, and it accounts for $\geq$ 5% of all inherited retinopathies and approximately 20% of children attending schools for the blind[3].

LCA is both genetically and clinically heterogeneous, and since 1996, ten genes/loci participating in a wide variety of retinal functional pathways have been implicated in the disease process[4]. LCA associated proteins participate in phototransduction (*GUCY2D*)[5], vitamin A metabolism (*RPE65*, *RDH12*)[6-8], photoreceptor development (*CRX*)[9], biosynthesis of cGMP phosphodiesterase (*AIPL1*)[10,11], photoreceptor cell development and structure (*CRB1*)[12-14] and disc morphogenesis (*RPGRIP1*)[15-17]. The discoveries of these genes has led to an increased understanding of the molecular determinants of retinal physiology/development by identifying new biochemical and cellular pathways. Therefore, LCA serves as a model for all human retinal dystrophies. The LCA genes at chromosomal loci 1p36[18], 6q11[19] and 14q24[20] remain to be identified. The seven currently known LCA genes account for ~40% of the cases[21], while genes underlying the remaining 60% of cases still await discovery. It is now evident that different LCA animal models respond to gene replacement therapy[22].

We previously found that obligate heterozygous parents of offspring with LCA mutations have unanticipated photoreceptor dysfunctions that appear to be specific for the involved gene, which points to the causal gene defect in the child, thereby significantly facilitating the molecular diagnostic process, while adding important functional information of the heterozygous mutations themselves. We documented cone ERG abnormalities in parents carrying *GUCY2D* defects[23], rod dysfunction in *AIPL1* carriers[24], normal rod and cone ERG function in the *RPE65* carriers[25], cone ERG dysfunction in *RDH12* carriers[26] and cone plus rod dysfunction in the *RPGRIP1* heterozygotes[27]. We confirmed many of these clinical findings by performing mutation-specific *in vitro* biochemical studies[28-30]. We also performed detailed clinical studies on another 31 obligate heterozygous parents of LCA children with known LCA mutations and found that more than 80% of them have mild gene-specific functional deficits and/or retinal abnormalities[25].

The purpose of the present study was to identify a possible distinctive phenotype of Heterozygotes for mutations in the Crumbs homolog-1 gene, *CRB1*. Recessive

mutations in *CRB1* are associated with a diversity of retinal phenotypes, including autosomal recessive retinitis pigmentosa type 12 (RP12)[12], Leber congenital amaurosis (LCA)[13,14] with or without the well-known retinal findings "preservation of the para-arteriolar retinal pigment epithelium" (PPRPE) and "Coats-like exudative vasculopathy." A dominant *CRB1* mutation has recently been associated with pigmented paravenous chorioretinal atrophy[31].

The *CRB1* protein is involved in photoreceptor development and photoreceptor structure and the two known *Crb1* knockout mice both show a distinctive inferior focal retinal dystrophy[32,33].

We tested the hypothesis that *CRB1* heterozygotes have a phenotype distinctive for *CRB1* defects. We specifically looked for regional retinal changes by multifocal ERG testing, which would correlate with the mouse *CRB1* phenotype. We observed regional areas of cone dysfunction in the carriers of *CRB1* mutations. We also identified an LCA child with a prominent maculopathy and postulated an *AIPL1* or *CRX* genotype. We were surprised to find a homozygous *CRB1* mutation, but further analysis also revealed a pathogenic *AIPL1* mutation. We here present clinical information on the patient's and the parent's phenotype, suggesting that the *AIPL1* defect contributed to the phenotypes as a modifier allele, while the homozygous *CRB1* mutation is causal.

Patients and methods

Clinical analyzes

Molecular diagnostic screening protocol: Our laboratory is involved with the molecular diagnostics of >400 LCA patients, and we have set up a protocol in which we postulate molecular hypotheses, based on the retinal and disease phenotype of the LCA patients. For example; an LCA patient with LP vision and relatively normal retinal appearance will be screened for *GUCY2D* mutations, while an LCA patient with measurable or improving vision, night blindness and translucency of the RPE, would suggest the *RPE65* gene for initial screening. LCA patients with macular changes (including macular colobomas) will be screened for *CRX* and *AIPL1* defects, while LCA patients with PPRPE are postulated to harbor *CRB1* mutations.

To investigate patterns of regional retinal dysfunction, we measured local electrophysiological responses of the retina, and chose the multifocal ERG (mfERG), as Vajaranant *et al.*[34] clearly demonstrated by mfERG that in female carriers of X-linked mutations, patchy areas of retinal dysfunction exist. The mfERGs were performed at two institutions; The Montreal Children's Hospital at the McGill University Health Center (MUHC) and at the Department of Ophthalmology of the University

of Illinois at Chicago (UIC) using a VERIS Science 5.1 system (EDI Inc., San Mateo, CA, USA). Both eyes were recorded while the pupil was undilated, and the stimulus matrix consisted of 61 hexagons scaled with eccentricity and was presented with a 7-inch monitor, measuring from 3-300 Hertz. This particular stimulus allowed us to record 61 localized cone ERGs from the macular and paramacular regions, subtending an area of up to 20° from the central fovea. The stimulus consisted of black and white hexagonal stimulus elements arranged concentrically around a fixation point. The stimulus was displayed on a digital camera and each element was reversed pseudorandomly in binary m- sequence at 75 Hz (frame rate). The stimuli were modulated between black (0.45 cd/m^2) and white (280 cd/m^2). Flash intensity was 1.028-log cd/sec/m^2, background intensity was 0.727-log cd/sec/m^2, while the fixation light measured 0.13-log cd/sec/m^2. Total recording time was 3 minutes and 38 seconds.

A total of seven obligate *CRB1* mutation carriers (from four LCA families) were ascertained for this study. Six carriers were investigated at the MUHC (Families I-III), and after we found abnormal focal mfERG measurements in four out of the six carriers, we wanted to confirm our results in a second, completely independent laboratory, with significant mfERG experience. We therefore recruited one individual from a *CRB1* pedigree with LCA children at the UIC, and did not share the details of our mfERG findings with the investigator. This one additional *CRB1* mutation carrier was studied in detail at the UIC (Family IV). We also repeated mfERG testing in our first subject (Family I:1, figure 5.1) to confirm our results. After each subject was counseled, informed consents were signed, approved by the Montreal Children's Hospital and the University of Illinois at Chicago institutional review boards. The study adheres to the declaration of Helsinki. Venous blood was drawn for DNA extraction and mutation analysis.

Detailed ocular and visual histories were obtained, pedigrees were drawn, and detailed eye examinations were performed on all seven carriers, including best corrected visual acuities by projected Snellen charts, cycloplegic refractions, slit lamp biomicroscopy, and dilated indirect ophthalmoscopy. Visual fields were measured by Goldmann kinetic perimetry, using the V4e and I4e test lights, moving the target from non-seeing to seeing retina. Cone and dark adapted mixed rod-cone electroretinograms (ERGs) were performed on both eyes and the a- and b-wave amplitudes and implicit times (peak times) were averaged in accordance with the standards recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV).[35] We then tested the following two hypotheses:
Hypothesis 1: Heterozygous carriers of *CRB1* mutations have regional retinal dysfunction on multifocal ERG (as suggested by the *CRB1* mice models) and
Hypothesis 2: A heterozygous *AIPL1* mutation acts as a modifier allele in a LCA patient with a severe maculopathy and a homozygous *CRB1* mutation.

Molecular analyzes

PCR amplification on genomic DNA (of LCA patients and parents) was performed with intronic primers designed to flank the splice junctions of each coding amplicon of LCA genes *CRB1* (12 exons; 27 amplicons), *AIPL1* (6 exons; 5 amplicons) and *CRX* (3 exons). Heteroduplex formation of the PCR products for dHPLC analysis was induced after heat denaturation and dHPLC analysis were performed with the WAVE DNA fragment analysis system (Transgenomics). The values of the buffer gradient, start and end points of the gradient and melting temperature prediction were determined by the WAVEMAKER software of Transgenomics. Variants in gel mobility identified by this technique were then subjected to bidirectional automated sequencing (ABI Prism, Applied Biosystems) on a model 3100 automated sequencer. Some of the *CRB1* mutations in this study were identified by a new LCA genotyping microarray, which now contains > 300 known LCA mutations from seven LCA genes (see Zernant *et al* for technical details)[21]. The LCA disease chip identifies currently known LCA mutations, while our conventional mutation screening methods identify and add novel changes. After we determined the *CRB1* mutations in the LCA children, we sequenced the appropriate *CRB1* amplicon of the parents DNA to assess the parental origin of each mutation. We used the following well-accepted set of five criteria to distinguish polymorphisms from pathogenic mutations. 1. The predicted effect of the base pair change on the protein product; 2. The relative frequency of the variation in LCA patients versus normal ethnically matched controls (> 1% in the controls will be assigned as a polymorphism); 3. Co-segregation of the mutant allele(s) in the affected families; 4. Homozygosity or compound heterozygosity in recessive LCA; and 5. Conservation of the codon across different animal species.

Results

Family I. In Family I, we identified a child with LCA who exhibited measurable vision and the striking PPRPE retinal pattern, which prompted us to screen the *CRB1* gene first and documented a c.2843G>A transition which predicts a p.C948Y mutation in *CRB1*, which we then determined was on the paternal allele (figure 5.1). We have not yet identified the maternal mutation, despite sequence analysis of the exons and flanking intronic sequences of the *CRB1* gene. The 35-year-old father complained about difficulties with vision in the right eye, but not the left. He denied nyctalopia and photo-aversion. Acuities were 20/20 OU with – 4.00 +3.00 x 120º OD and – 1.75 OS. Retinal examinations were positive for marked narrowing of the retinal blood vessels in both eyes. The retina was otherwise normal. Average cone b-wave amplitudes were slightly decreased compared to normal at 92 µV with a 30 ms implicit time, while the averaged mixed rod and cone b-wave amplitudes were markedly decreased at 142 µV (normal > 220 µV), with a normal 50 ms implicit time (normal is 45 +/- 3 ms). The mfERG of the right eye showed a striking superior,

regional pattern of abnormality in the right eye (figure 5.1), which corresponds to inferior retinal dysfunction, while the left eye appeared entirely normal (figure 5.1). The blue color superiorly indicates decreased retinal sensitivity, which is confirmed by the raw data showing the actual waveforms and decreased heights of the waves. The central peak corresponding to the foveal input is also lower in the right eye. We averaged the superior and inferior mfERG field data and used Student *t* testing to determine statistical differences. We found an average of 13.796 nV/deg^2 superiorly (5.85 nV SD) and 23.44 nV/deg2 (7.08 SD) inferiorly. The two means are significantly different ($p<0.001$). We repeated the mfERG on this subject two years later and confirmed the results (data not shown). The carrier mother was asymptomatic, had a completely normal retinal examination, and both the standard ERG (cone and rod signals) and the mfERG were normal. There was no superior mfERG field abnormality in either eye (figure 5.1).

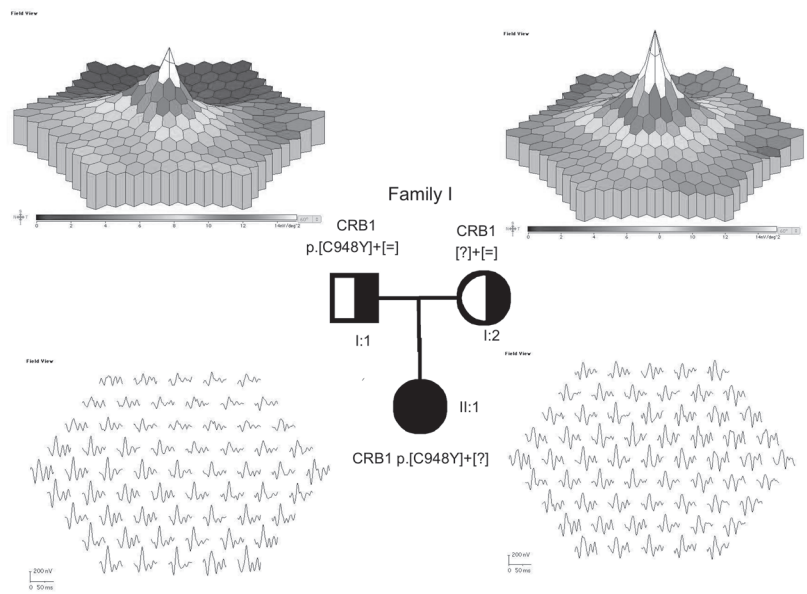

Figure 5.1 Pedigree, *CRB1* mutation and mfERGs of Family I
Patient II-1 carries a paternal p.C948Y *CRB1* mutation (noted as p.C948Y for ease of illustration). The paternal mfERG of the OD is shown on the left with the 3D image at the top and the raw data at the bottom, both showing the superior defects, corresponding to the inferior retina. The maternal mfERG of the OD is on the right and both the 3D and raw data show normal values.

Family II. In the second LCA family, the affected LCA patient also had the PPRPE retinal disease pattern and we again postulated *CRB1* involvement. We then determined that the child harbored a c.493_501del 9 bp that predicts a deletion of three amino acids p.D165_I167del. We also found a c.1360G>A transition, which predicts a p.G454R missense mutation in *CRB1* (figure 5.2). We then documented that both the p.D165_I167del and the p.G454R mutations were on the paternal allele. The *CRB1* mutation on the maternal allele has not yet been identified, despite sequence analysis of the exons and flanking intronic sequences of the *CRB1* gene. The 46-year-old mother was asymptomatic and had a normal retinal exam. We measured averaged cone b-wave amplitudes of 100 μV (N>120 μV), which is slightly below normal with a 33.5 ms implicit time. Averaged mixed rod-cone b-wave amplitudes were normal at 239 μV (N>220 μV) with a normal 39.5 ms implicit time. The mfERG of the mother were abnormal for both eyes and showed a regional pattern of abnormality with a marked lowering of the central peak (figure 5.2, only the right eye is shown). The father was also asymptomatic and had a normal retinal exam as well. His cone b-wave amplitudes averaged 98 μV, which is again slightly below normal with a 32.3 ms implicit time, and his mixed rod-cone b-wave amplitudes averaged 223 μV with 40.5 ms implicit times. MfERGs of both eyes showed regional field abnormalities associated with a marked decrease in central sensitivity, less obvious than the mother (figure 5.2, only the right eye is shown).

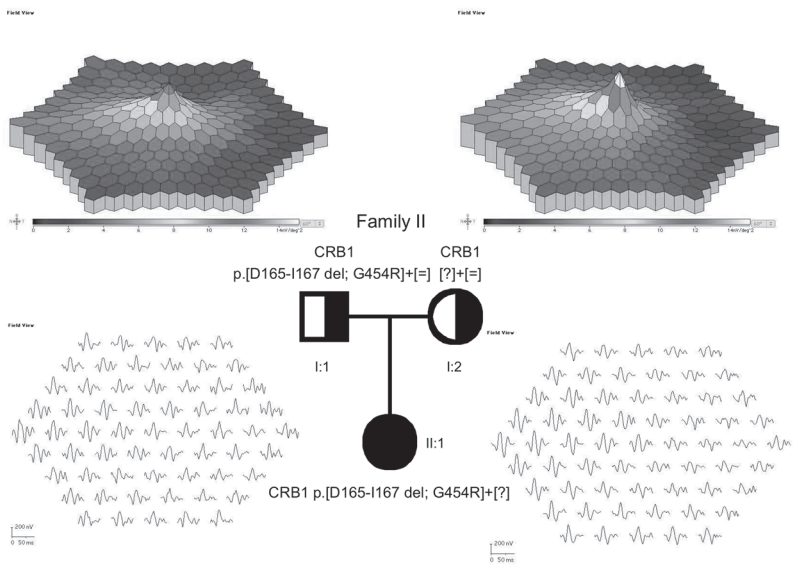

Figure 5.2 Pedigree, *CRB1* mutation and mfERGs of Family II
Patient II-1 carries the paternal p.D165-I167del and p.G454R *CRB1* mutations. The paternal mfERG data of the OD is on the left, the maternal mfERG data of the OD is on the right, both showing the superior mfERG abnormalities. The abnormalities are especially obvious when comparing the plots to normal plots such as displayed on the right side of figure 5.1.

Family III. In the third family (figure 5.3) we diagnosed a child with LCA and a prominent bilateral maculopathy (figure 5.5), and initially postulated sequence variants in *AIPL1* or *CRX*, but not *CRB1*. There was no PPRPE and there were no intraretinal white dots. Our genetic analysis revealed a well-known and previously reported heterozygous c.905G>T transversion which predicts a p.R302L *AIPL1* mutation, while no mutations were found in *CRX*. In our comprehensive screening protocol, we were surprised to also find a homozygous c.1345C>T transition, which predicts a p.Q449X nonsense mutation in *CRB1*. We then confirmed that both parents carried the p.Q449X *CRB1* mutation heterozygously and then determined that the father also carried the p.R302L *AIPL1* mutation. Both parents were asymptomatic, had normal dilated retinal examinations, and both mixed rod-cone and isolated cone signals were normal on conventional ERG (mixed rod-cone b-waves > 220 μV; cone b-waves > 120 μV). The mother's mfERG appeared to be normal (figure 5.3), while the father had substantially abnormal recordings on the mfERG, with superior, temporal and inferior field abnormalities, associated with a marked decrease in central sensitivities (figure 5.3).

Because of the potential importance of our previous findings, we wanted to confirm our results in a parallel study. We identified a fourth *CRB1* family at UIC and performed detailed mfERG studies without prior knowledge of the detailed findings.

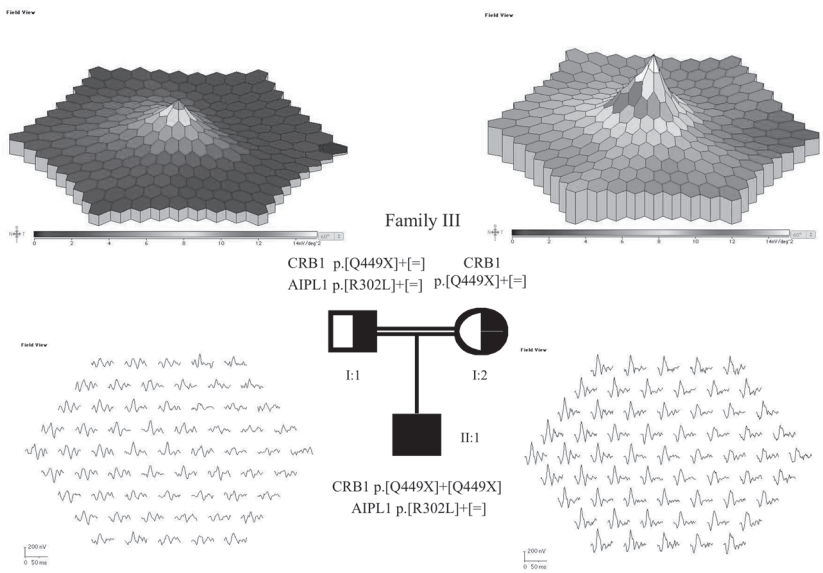

Figure 5.3 Pedigree, *CRB1* and *AIPL1* mutations and mfERGs of Family III
Patient II-1 carries the homozygous p.Q449X mutations in *CRB1* and the additional paternal p.R302L *AIPL1* mutation. The paternal mfERG data of the OD is on the left showing striking abnormalities, while the maternal mfERG of the OD on the right appears normal.

Family IV. In the fourth family, we found that three offspring had LCA, with retinal pigmentary changes and white intraretinal dots, but without PPRPE. No molecular hypothesis could be postulated, and all LCA genes were systematically screened. The LCA proband was found to have a c.1463T>C transition which predicts a p.F488S and a c.2258T>C transition which would lead to a p.L753P missense mutation in *CRB1* and a c.1301C>Transition which would lead to a p.A434V mutation in *RPE65* (figure 5.4). We traced the p.L753P change in *CRB1* and the p.A434V mutation in *RPE65* to the maternal allele and assumed that the p.F488S mutation in *CRB1* came from the paternal allele, as he was deceased prior to our studies. The p.A434V allele is found in 11% of the black population (Dr. EM Stone, personal communication), we therefore consider this change a non-disease causing polymorphism. The carrier mother was found to be asymptomatic and had a normal dilated retinal exam. Her mfERG recordings were, however notably abnormal. The right eye was found to have a marked regional mfERG field abnormality, associated with a marked decrease in the sensitivity of the fovea, while the left eye was entirely normal (figure 5.4). The raw data show a similar pattern.

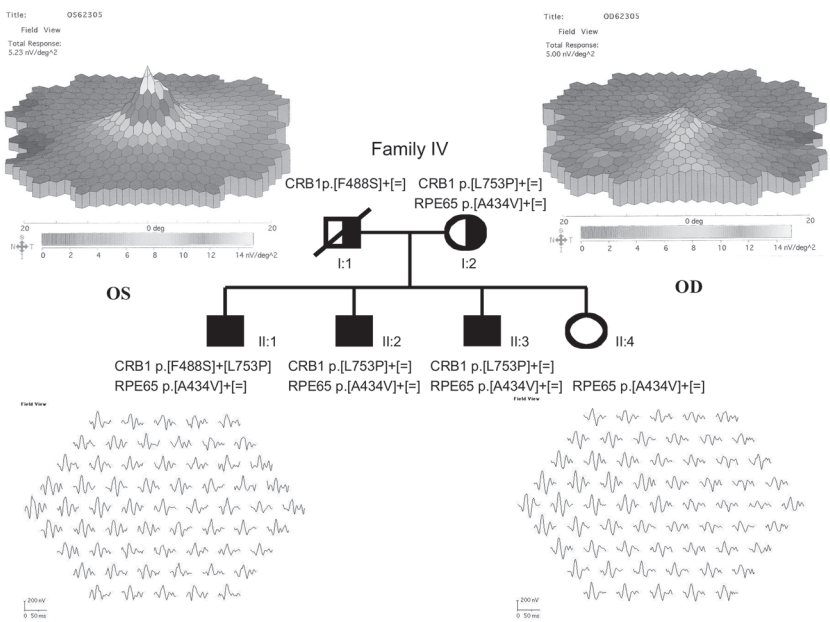

Figure 5.4 Pedigree, *CRB1* and *RPE65* mutations and mfERGs of Family IV
Patient II-1 carries the p.F488S and p.L753P mutations in *CRB1* with the additional maternal p.A434V variation in *RPE65*. Only the maternal mfERG data is shown, as the father was unavailable for analysis. On the left is the left eye (OS) with the 3D image above and raw data below which appear normal, while on the right is the right eye (OD) showing abnormal mfERG patterns predominantly in the superior region on both the 3D image (above) and raw data (below).

Child with LCA and a maculopathy

The child with LCA from Family III was first seen by us at the age of three years, and was diagnosed with congenital blindness and nystagmus elsewhere. He was a term baby from a consanguineous couple from Lebanon. On our first exam we found that the child was able to fix and follow. His cycloplegic refractions were –0.5 OD and +1.50 OS. He was also found to have rotatory nystagmus, paradoxical and amaurotic pupils, a large exotropia, and the oculodigital sign of Franceschetti. He much preferred day vision and protested when the lights were turned off. Averaged cone b-wave amplitudes were 15 µV (N > 120 µV) with 30 ms implicit times, while the mixed rod-cone b-wave was non-detectable. The retinal exam was striking (figure 5.5a and 5.5b). The optic discs were normal, the retinal vessels were narrow and there was an absence of pigmentary degeneration and PPRPE. Both maculae were abnormal with multiple, similarly sized, cystic white lesions with surrounding black rims (total size, approximately four disk diameters) were observed (figure 5.5a and 5.5b). At age 5 we found that the cycloplegic refractions had become +2.00 + 1.00 x 90 OD and + 4.50 OS and the acuities were measurable at 6/200 OU. We postulated involvement of the *AIPL1* or *CRX* gene, as these two LCA genes are associated with macular colobomas and/or maculopathies. DNA analysis revealed a heterozygous p.R302L *AIPL1* mutation and an additional homozygous p.Q449X mutation in *CRB1*.

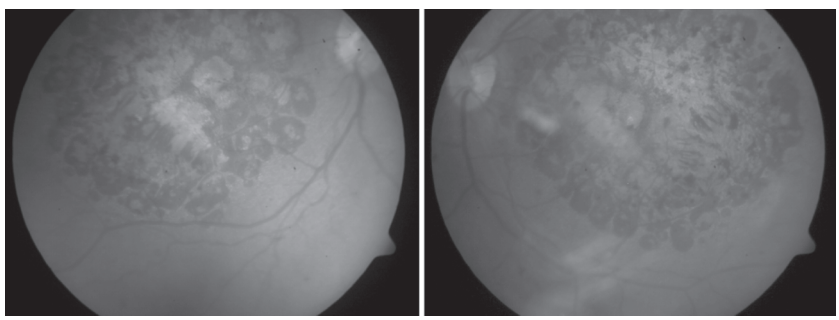

Figure 5.5a + b. Fundus pictures of Family III LCA patient II-1
Shown are the posterior poles of the retinas of the right (5a OD) and left eyes (5b OS) of the affected LCA child with the homozygous p.Q449X mutation in *CRB1* and a heterozygous p.R302L mutation in *AIPL1*. Note the conspicuous maculopathy.

Discussion

Human photoreceptors are highly specialized and polarized cells which, in the outer nuclear layer (ONL), are in close contact with Müller glial cells for structural and metabolic support. The adhesion belt named the outer limiting membrane (OLM) contains multiple adherence junctions, which are present between photoreceptors and Müller cells. These adherence junctions consist of multi-protein complexes and are linked to the cytoskeleton of the cell. A core component of these complexes is the trans-membrane protein Crumbs homolog-1, *CRB1*[32,33].

A natural *Crb1* mutant mouse, called retinal degeneration 8 (*rd8*)[32] was found to harbor a homozygous single base pair deletion in *Crb1* (c.3481delC) causing a frameshift and an early stop codon and likely leads to nonsense-mediated decay of the encoded RNA. The *rd8* mouse develops severe retinal abnormalities, including focal photoreceptor degeneration and irregularities of the OLM. Mehalow *et al* found that the *rd8* mice developed irregular shaped large white subretinal spots detectable at the age of 3 weeks, which were more heavily concentrated in the infero-nasal quadrant of the retina. These spots were then found to correspond histologically to retinal folds and pseudo-rosettes. They also found OLM fragmentation, outer segment shortening, but normal inner segments. By 5 months, the outer segments had virtually disappeared, the inner segments were swollen, and the Müller cell processes were unusually prominent. The retinal degeneration was unexpectedly *focal* in appearance, with nearly normal retina present at the edge of a region with severe degeneration. The reason for this is still unclear. The photoreceptor dysplasia and degeneration reported by Mehalow *et al* in the *rd8* mouse with the *Crb1* mutations strongly vary with the genetic background, suggesting modifier effects from other retinal genes[32].

Van de Pavert *et al* inactivated both *Crb1* alleles to produce a complete null and examined the resulting mouse retina[33]. There were marked differences between the *Crb1*$^{-/-}$ and the *rd8* retinal findings. Neither two week nor 2-month-old *Crb1*$^{-/-}$ mice had retinal abnormalities. At 3 months however, the mice developed focal areas of retinal degeneration, while the OLM was ruptured, and there was protrusion of single or multiple photoreceptors through the OLM into the subretinal space. Also, there was ingress of photoreceptors into the inner retina, namely the outer plexiform layer. One of the most striking histological findings was a double layer of photoreceptors (half rosettes). These rosettes developed normal inner segments and a full OLM, very much unlike the *rd8* model. This finding suggests that *CRB1* is not essential for the formation of junctional complexes and OLM, but rather for the maintenance of these structures. In six-month-old *Crb1*$^{-/-}$ mice large ectopic photoreceptor layers were identified, which were so large that they resembled a ”funnel” abutting the ganglion cell and inner limiting membrane. The authors suggest that the initial insult of the *Crb1* mutation is the loss of the photoreceptor to Müller glial cell adhesion at retinal foci. Light exposure experiments revealed a significant increase

in retinal degeneration in the *Crb1*$^{-/-}$ mouse especially infero-temporally; therefore light enhances the retinal degeneration in the *Crb1*$^{-/-}$ retinas[33]. These histological abnormalities in the two *CRB1* mice models prompted us to hypothesize that regional retinal abnormalities exist in carriers of *CRB1* mutations, and that led to this study and the surprizing mfERG abnormalities.

As we have thus far found LCA gene-specific phenotypes in obligate heterozygotes with mutations in *GUCY2D*[23] and *RDH12*[26] (cone ERG dysfunction), *RPE65*[25] (normal ERGs), *AIPL1*[24] (rod ERG dysfunction), and *RPGRIP1*[27] (rod plus cone ERG dysfunction), we wanted to know if *CRB1*-specific changes occur as well, that may be useful in our classification system designed to use simple clinical tests on carriers to point to the molecular defect in the blind child or might in the future help to identify carrier states in family members of LCA patients. We now report that five out of seven *CRB1* mutation carriers from four tested families were found to have novel and distinctive retinal dysfunction by multifocal ERG testing. We documented superior mfERG abnormalities in one carrier (Family I-1 in figure 5.1), supero-temporal mfERG abnormalities in three carriers (Family II-1 and II-2 in figure 5.2 and Family IV-2 in figure 5.4), and supero-temporal-inferior mfERG field abnormalities in one carrier (Family II-1 in figure 5.3). We also found that all five patients with regional mfERG abnormalities had a co-existent central abnormality, which represents the signal from the fovea.

Abnormalities were detected irrespective of the type or severity of the *CRB1* mutation, as we found similar changes in the carriers of a complex mutation consisting of a missense mutation (p.G454R) with an in-frame deletion (p.D165_I167del; Family II), a nonsense mutation (Family III), and missense mutations (Families I and IV).

We found however, that not all seven of the *CRB1* mutation carriers exhibited a regional retinal dysfunction on mfERG testing, as two of the carriers had normal recordings. We also found striking asymmetries between eyes of two of the carriers, who showed the mfERG abnormalities in one eye, but not in the other. We do not yet know the reason for this, but we postulate that other genetic and environmental factors may contribute to the *CRB1* carrier phenotype. We found that most *CRB1* carriers in this small series had normal or mildly abnormal rod and cone full field ERGs except one carrier that exhibited significant rod ERG defects (Family I). This is the same carrier who also had obvious narrowing of the retinal blood vessels and was the only carrier who was symptomatic. The rest of the carriers were asymptomatic and had normal retinal examinations including normal vessel caliber. We postulate that the mfERG changes we have documented in the carriers may be caused by retinal folds and/or pseudorosettes (as was found in the mice), as a result of a disrupted OLM and that light toxicity may play a role in the carrier phenotype, as it has been documented in the *Crb1* knockout mouse[32]. It will be very informative to perform *in vivo* high-resolution microscopy (OCT-3) in the *CRB1* mutation carriers in future studies to test this hypothesis as Jacobson *et al* documented thickened

retinas in affected individuals with LCA and *CRB1* mutations[36]. We also plan to perform further psychophysical testing by automated visual field analysis to extend our mfERG findings in the *CRB1* carriers.

In terms of genetics of the LCA patients and the co-segregation of their *CRB1* mutations, we found that only Family III shows definite autosomal recessive inheritance of the *CRB1* mutations, with a possible modifier effect from *AIPL1*. In Family IV, only II.1 follows an autosomal recessive pattern of *CRB1* variants. The other two affecteds in Family IV carry two maternal variants. These patients and the patients of Families II and I could be also be digenic. In three of the four families reported here, the segregating *CRB1* variants therefore do not fully explain LCA in the younger generations. In Families I and II, the maternal *CRB1* variants could not be detected; in family IV, two out of three patients do not carry maternal and paternal *CRB1* variants. Thus, it is possible that defects in other genes cause, or play a major role in, the phenotype of these LCA patients. However, in view of the very low incidence of *CRB1* variants in the general population (heterozygosity frequency between 1/350 and 1/500) (assuming an incidence of LCA between 1/50.000 and 1/100.000, and a *CRB1* share of 10%), the identification of *CRB1* variants in these families (from a total of 250 LCA patients investigated) hardly can be coincidental. Thus, we think that the *CRB1* variants identified in this study, either in combination with as yet unidentified *CRB1* defects, or in combination with defects in other genes, are causal for LCA in the patients of Families I and II and for two of three affected siblings in Family IV. As such, we cannot exclude the possibility that mfERG abnormalities in parents of LCA patients are not only due to heterozygous *CRB1* variants, but, as exemplified by parent I.1 of Family III and parent I.2 of Family IV, are due to defects in more than one gene.

In the second part of our study, we identified a homozygous *CRB1* mutation p.Q449X associated with a retinal LCA phenotype consisting of a prominent and unusual maculopathy, which to the best of our knowledge, has not previously been described for *CRB1* patients[24]. We wanted to test the hypothesis that *CRX* or *AIPL1* defects contributed to this phenotype as modifier alleles, as both *CRX* and *AIPL1* are well known to be associated with maculopathies in LCA[24].

In an isolated Dutch population, we previously documented intra-familial phenotypic variability (both in severity and progression of disease) in patients with early-onset severe retinal dystrophy, and determined that 13 of the 14 affected members share a homozygous p.Y368H mutation in *RPE65*[37]. Despite sharing an identical genotype, we made the surprising observation that the members did not share a common phenotype, as we carefully documented three disparate disease types in terms of their visual function: some patients deteriorated, some improved, while yet others remained stable[37]. Identical genotypes with disparate disease phenotypes in this and other families led us to hypothesize the existence of modifier genes/alleles. We tested this idea with a new LCA microarray in a cohort of 200 LCA patients and

found that 15% of patients have three sequence variants in two genes, instead of the expected two in the same gene[21]. We found that the phenotype was more severe in patients with three sequence variants in five families, which were available for meaningful functional comparisons.

In our LCA patient with homozygous *CRB1* mutations and with an unusual prominent maculopathy, we also identified a heterozygous p.R302L in *AIPL1* and postulate that the LCA is caused by the homozygous *CRB1* defects and that the *AIPL1* mutation causes the altered phenotype (maculopathy), and we provide three lines of suggestive evidence for this in view of biochemical analysis, which are not yet completed. Firstly, a small diffuse maculopathy has only been reported once in a patient with *CRB1* mutations p.C948Y + T745M, but this macular lesion is unlike the maculopathy of our patient[38]. Second, additional support for an effect of the p.R302L mutation in *AIPL1* comes from comparing the phenotypes of the two carrier parents, the father with the *CRB1* p.Q449X and *AIPL1* p.R302L and the mother with only the *CRB1* p.Q449X mutation. The mfERG data strongly suggest, that the cone dysfunction in the father is significantly worse than that of the mother (or any other carrier in this study). Third is the fact that the p.R302L in *AIPL1* has been found to have a biochemical abnormality in *in vitro* studies with *NUB1*, the molecular partner of *AIPL1*[39].

The p.R302L mutation in *AIPL1* was initially reported by Sohocki *et al* and lies just outside the tetra-tricopeptide region (TPR) 3 domain (3') of the *AIPL1* protein in the proposed *NUB1* binding area, which spans codons 181-330 of *AIPL1*[40]. The p.R302L mutation was found to inhibit immunoprecipition between the mutant *AIPL1* and endogenous *NUB1*[39]. The p.R302L mutation however was found not to affect the interaction between *AIPL1* and farnesylated proteins[10]. Our data suggests that the *CRB1* associated phenotype was altered by the p.R302L allele found in *AIPL1*.

In summary, we found that the heterozygous phenotype of carrier parents with children who have LCA caused by *CRB1* mutations do not have a normal phenotype as suggested by classical Mendelian inheritance models. Instead, we document a novel, unique retinal phenotype of the obligate carriers with *CRB1* mutations consisting of regional retinal dysfunction (found by mfERG testing), which corresponds to abnormalities in the opposite mfERG field. This correlates well with both published *CRB1* knockout animal models. We also present a LCA retinal phenotype with homozygous nonsense mutations in *CRB1* and with a prominent macular lesion and suggest a modifier effect by a heterozygous p.R302L *AIPL1* allele.

Acknowledgements

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6 Discussion

Discussion

The most frequently occurring human eye diseases in the Western world have a multifactorial aetiology and as a group they are relatively common. Age-related macular degeneration (AMD), for example, is the leading cause of irreversible blindness in the Western world, affecting approximately 30% of people over the age of 75[1]. Monogenic retinal dystrophies, on the other hand, are far less common with an incidence of approximately 1 in 2000[2]. These inherited retinal diseases often have an early onset and thereby have a major impact on future prospects concerning education and employment of young patients.

The retinal dystrophies discussed in this thesis are arRP, arCRD, LCA, and STGD1. Many causative genes acting in different pathways, have been identified for each of these retinopathies, with the exception of STGD1, for which mutations are confined to the *ABCA4* gene. In the near future, it will become increasingly important to determine the molecular causes underlying these diseases, as a molecular diagnosis may form the basis for accurate patient counselling including therapeutic advises. A molecular diagnosis in every patient with an inherited retinal disease can only be achieved by expanding the knowledge on the genes involved, the pathogenicity of individual mutations, the genotype-phenotype correlations, and the integration of this knowledge. The overall goal is to provide a classification framework for clinicians and vision scientists that contains all mutational and detailed phenotypical attributes.

The main results of the studies presented in this thesis regarding molecular and clinical results on inherited retinal diseases, will be discussed below.

The role of *ABCA4* in arCRD and arRP

ABCA4 plays a key role in the visual cycle and mutations in the *ABCA4* gene were previously found to underlie STGD1, a significant portion of the arCRD patients, and a small percentage of atypical arRP cases[3-5]. Furthermore, it is a susceptibility locus for AMD[6]. Since these diseases are very different in clinical severity, a genotype-phenotype model was proposed in which the amount of residual ABCR protein activity was inversely correlated to the severity of the retinal dystrophy[7].

In chapter 2.1, a new genotyping technology was used to detect *ABCA4* sequence variants in a large cohort of CRD and RP patients. Based on these results we estimated that a small percentage of the arRP patients carry *ABCA4* mutations. On the other hand, 33% of the CRD patients in our cohort showed mutations in *ABCA4.* Although this result is consistent with earlier studies (chapter 2.1 and references therein), this may well be an underestimate since the APEX genotyping array used for mutation screening can only detect previously identified mutations,

and subsequent single strand conformation polymorphism (SSCP) testing was only conducted when at least one *ABCA4* mutation was detected upon microarray analysis. Furthermore, based on extensive mutation analysis of STGD1 patients, it is estimated that 60% of the *ABCA4* mutations are currently known[8].

ABCA4 is the only arCRD gene identified at the moment. Furthermore, arCRD loci are mapped at 1q11.2-q14 (CORD8)[9] and 8p11 (CORD9)[10]. ArCRD patients with two *ABCA4* mutations do not show distinctive phenotypic characteristics in comparison to arCRD patients without mutations in *ABCA4*. Therefore, a clear-cut genotype-phenotype correlation for the involvement of *ABCA4* in arCRD cannot be made and an accurate estimate for *ABCA4* involvement in arCRD is complicated. Sharon and coworkers[11] identified *RPGR* mutations in 5 of 11 male CRD patients who had no female siblings with RP. Among the CRD patients of our cohort without *ABCA4* variants, 62% are male. Hence, it is possible that several male CRD patients actually carry mutations in *RPGR* or *RP2*, the two xlRP genes.

It can be concluded that mutation screening of the *ABCA4* gene is worthwhile in arCRD and isolated CRD cases. A rapid, efficient and affordable first screening method would be the ABCR500 APEX microarray[8]. In this way, approximately 60% of the alleles are found in patients with STGD1 belonging to the Caucasian population. As sequence analysis yields ~80% of alleles[12], 75% of the theoretically expected alleles can be found this way.

The Affymetrix arRP Resequencing Array[13] identifies all exonic *ABCA4* single nucleotide substitutions and deletions. In addition, 5 base pairs of flanking intronic sequences are analysed. Heterozygous deletions larger than one nucleotide, as well as all duplications, are not detected on this screening platform since their inclusion would increase the number of oligonucleotide probes on the microarray tremendously. Approximately 15% of all *ABCA4* variants are abnormalities of this kind, which will be missed if present heterozygously (as most variants are in *ABCA4*). Hence, the resequencing microarray can be predicted to identify approximately 65% of the theoretically expected alleles. This disadvantage can be partially overcome by including oligonucleotide probes on the resequencing array that specifically detect the known deletions and insertions. The small extra efficiency of the *ABCA4* resequencing microarray compared to the APEX technology does not outwage the much higher (~10-fold) costs involved. Capillary-based heteroduplex analysis or direct sequence analysis can be used to identify *ABCA4* variants in patients with one or no mutation.

In contrast to arCRD, we showed that mutations in *ABCA4* are responsible for just a minor fraction of all arRP cases. The arRP phenotype associated with *ABCA4* mutations is relatively specific and is usually characterised by an early-onset pan-retinal dystrophy with a severe and highly progressive nature. Patients lose both central an peripheral vision before the age of 40 years. We showed a similar

phenotype in older arCRD patients (chapter 2.2). The arCRD patients showed pale optic discs with severely attenuated vessels and severe atrophy of both the posterior pole (maculopathy) and the midperiphery with extensive bone-spicule pigmentations throughout the whole retina. An association with nystagmus and atrophic nummular areas in the macula was also shown.

In patients with an early-onset, severe and highly progressive arCRD or arRP, *ABCA4* is a good first candidate gene to screen. However, no systematic analysis of arRP patients has yet been performed to validate this assumption. Again the ABCR500 APEX microarray[8] is a good, relatively cost-effective, first pass screening tool for mutation analysis.

The identification of patients with arCRD or arRP carrying *ABCA4* mutations might become of greater importance since some therapeutic trials look promising. *Abcr* knockout mice were shown to accumulate lipofuscin pigments[14-16]. The major fluorophore of lipofuscin is *N*-retinylidene-*N*-retinylethanolamine (A2E)[17] of which the biosynthesis is dependent on circulating retinol (vitamin A). A2E is cytotxic to RPE cells in several different modes. Thus, therapeutic strategies were developed to target the biosynthesis of A2E and its accumulation. Furthermore, it was proven that the biosynthesis of A2E and its derivates are illumination dependent[18]. *Abcr*-/- mice raised in total darkness showed complete inhibition of biosynthesis of A2E and therefore patients with *ABCA4* mutations may possibly benefit from reduced light exposure, for instance by wearing sunglasses or dark contact lenses. Pharmaceutical interventions are also under investigation. Isotretinoin (a drug used in humans for acne treatment) was tested as a competitive inhibitor of the synthesis of 11-*cis*-retinal dehydrogenase by lowering the levels of *at*RAL (primary reactant in A2E biosynthesis)[14]. The retinal degeneration was prevented successfully in an animal model[19]. However, the doses used in these mice were far higher than acceptable for humans (systemic toxicity). Alternative therapeutic interventions based on the modulation of intracellular retinoid concentrations are currently ongoing, for instance with *N*-(4-hydroxyphenyl)retinamide (HPR)[20].

Leber congenital amaurosis: genotype-phenotype correlations

A cohort of 58 Dutch and Belgian LCA patients was genotyped using a newly developed LCA APEX microarray (chapter 3), and mutations were detected in 33% of the cases. In a study performed by Hanein and co-workers, a larger number of LCA patients was screened using a more comprehensive but much more labor intensive approach (linkage analysis, dHPLC, direct sequencing), which revealed mutations in 44% of the cases[21]. Assuming that 44% is the maximum percentage of patients in which variants can be found today, we can conclude that the LCA APEX microarray detected 75% of the expected mutations.

In our patient cohort, mutations were most frequently identified in the *CRB1* gene (16%) and all carried either the p.C984Y or the p.K801X mutation. The second most frequently involved gene was *GUCY2D.* The *GUCY2D* p.R768W mutation appears to be a founder mutation in the Northwest of Europe, because of it unusually high frequency. In previous studies, mutations in *CRB1* were found to underlie 10-13% of the LCA cases[21-23]. Recently, den Hollander and co-workers analysed 24 French-Canadian LCA patients and found only one mutated allele[22]. On the other hand, a Spanish study revealed *CRB1* to be the causative gene in ~2/3 of all LCA patients with proven mutations, suggesting that the *CRB1* gene is a major LCA causing gene in Western Europe (Vallespin E, personal communication, 2005). The latter two studies illustrate extensive ethnic and geographical differences in the occurrence of *CRB1* mutations.

In chapter 3 specific genotype-phenotype correlations were made for three LCA genes. Patients with *AIPL1* mutations showed very poor visual acuity and hypermetropia with essentially normal appearing fundi on initial examination. After the age of eight years, extensive pigmentations became present with a striking bulls' eye maculopathy. All patients with *CRB1* mutations showed some or all signs of the RP12 phenotype, being relatively normal appearing optic discs and vessels, a distinct maculopathy, a nummular type of pigmentations, yellow-white subretinal dots, PPRPE, Coats-like exudative vasculopathy and vascular sheathing. LCA patients with *GUCY2D* mutations showed an extremely severe phenotype of no light perception and relatively normal appearing fundi until at least the age of 30 years.

In a relatively large LCA cohort, Hanein and co-workers suggested that symptoms such as night blindness and photophobia may be distinctive symptoms that point to the gene involved[23]. Our study did not confirm this correlation. Night blindness or photophobia as gene-specific characteristics may be applicable to a group of patients with a certain genotype; however they cannot be used as good predictors in the individual patient. Unfortunately, although we presented specific genotype-phenotype correlations, it may still be very difficult to predict the gene involved in a single LCA patient after one clinical examination. Our study, although small in size, did indicate that there are trends in the correlation between LCA gene defects and the resulting phenotype. This correlation likely becomes more complete in the near future, as more detailed structural (optical coherence tomography) and functional (autofluorescence) imaging techniques are now available. These results, together with a longer follow up of the LCA patients, will add new phenotypic aspects to the genotype-phenotype correlations. In the future, more genotype-phenotype studies need to be performed in order to establish better indicators for which specific gene is involved.

In chapter 4 the gene encoding the retinal pigment epithelium-specific 65-kDa protein (RPE65) was studied. *RPE65* is the only gene studied in this thesis that is expressed exclusively in the RPE. The most detailed phenotypic description of

patients suffering from autosomal recessive early-onset retinal dystrophy caused by *RPE65* mutations consistently showed preserved vision in childhood[24-26] with a useful visual function beyond the second decade of life[27], which later declined. Interfamilial variations in the *RPE65*-associated phenotype were found in these studies, which were attributed to the effects of different types of mutations (different amount of residual protein function).

In chapter 4 we reported on the existence of intrafamilial variations in patients with the same homozygous mutation in *RPE65*. In a large, previously isolated Dutch population a p.Y368H *RPE65* mutation was detected homozygously in 13 out of 14 patients from 10 related families. The patients all showed signs of night blindness, nystagmus, severe visual impairment with relatively well-preserved visual fields and a non-detectable ERG in early childhood. On fundoscopy, the older patients showed optic disc pallor, attenuated vessels and a hypopigmented periphery. Surprisingly, a wide range of visual acuity (VA) and natural history of visual function was observed. Approximately one third of the patients showed mild improvement, one third showed deterioration and one third showed a stable VA over a mean 9-year follow-up period. Clearly, other genetic and/or environmental factors must be involved in the heterogeneous phenotype in this pedigree.

Further studies of this population revealed the founder mutation heterozygously in 1/28 of healthy controls rendering a risk of 1 in 56 for the child of a patient with a partner from this population to develop the retinal dystrophy due to homozygous *RPE65* mutations. Likewise, heterozygous carriers with partners from this population have a risk of 1 in 112 that any of their children will develop the disease. Recently, additional patients from this population with either arRP or early-onset RP were ascertained. Sequence analysis of the *RPE65* gene revealed new mutations in this gene, rendering the relative burden of *RPE65* mutations in this population even higher (S. Yzer, F.P.M. Cremers, unpublished data). Our studies now enable p.Y368H carrier detection in this population, which theoretically could lead to a significant decrease of children born with this severe sensory handicap.

It is estimated that autosomal recessive mutations in *RPE65* account for 6-16%[24,28,29] of the LCA cases in the world. The identification of patients with *RPE65* defects has become very important since gene therapy studies in *RPE65* deficient animal models proved to restore photoreceptor function both on observational and functional (ERG) studies[30-32]. A human clinical trial for single dose subretinal delivery of recombinant adeno-associated virus carrying wild-type *RPE65* cDNA, is not far off.

As illustrated in our studies, LCA is a remarkably heterogeneous disease, both clinically and genetically. Genotyping-phenotyping efforts as presented in this thesis will ultimately lead to a more detailed knowledge facilitating phenotypical differentiation. Together with new technologies these efforts will facilitate and speed up the molecular diagnostic process. Unfortunately, the current knowledge on LCA

phenotypes does not provide enough information for accurate gene selection in individual patients. In order to aid a more profound genotype-phenotype correlation in the future, more mutations need to be identified, other LCA genes need to be identified and additional genotype-phenotype correlation studies are needed. Until then, the most logical and most cost-effective genotyping approach would be to have a patients' DNA tested using the APEX LCA microarray.

Carriers of LCA mutations show distinctive functional abnormalities

Classical genetic definitions (Mendelian inheritance) teach that recessive mutations only cause a phenotype in the individual that carries two defective genes. A carrier with only one copy of the defective allele is thought to be protected by the wild-type allele and therefore show no obvious disease signs. Obligate heterozygous carriers of LCA mutations in the *AIPL1, GUCY2D, RDH12* and *RPGRIP1* genes, however, were shown to have distinct ERG abnormalities[33,34], without any other signs or symptoms. Carriers of *AIPL1* mutations showed abnormal rod amplitudes[33] whereas carriers of heterozygous *GUCY2D* pathogenic sequence changes showed cone abnormalities, as did carriers of *RDH12* variants. *RPGRIP1* heterozygotes showed both rod and cone ERG dysfunction[34]. In contrast to carriers of *AIPL1*, *GUCY2D*, *RDH12* and *RPGRIP1* pathogenic sequence changes, the heterozygous carriers of *RPE65* mutations show normal ERG function[35-37]. Apparently, the presence of only one wild-type LCA allele in most families is not enough to suppress a (sub)clinical phenotype. Our goal in chapter 5 was to elucidate the possible *CRB1* heterozygous phenotype.

Two different Crb1 animal models were previously studied. In a natural Crb1 mutant mouse, the predicted Crb1 protein is truncated lacking the transmembrane and intracellular domain. Histologically, these animals show irregularities at the outer limiting membrane and loss of the photoreceptors, eventually resulting in multiple intraretinal pseudorosettes localized to the inferior nasal quadrant of the fundus[36]. In the second mouse model both *Crb1* alleles were inactivated by deleting a 2.9 kb segments of genomic *Crb1* sequence containing the upstream promoter region, exon 1 (encoding the start of the protein) and part of intron 1. This model shows giant half rosettes in the inferior temporal quadrant but no loss of overall retinal function on ERG [37].

In chapter 5 obligatory carriers of *CRB1* mutations were tested for gene specific electroretinography abnormalities. Since the retinal dystrophies in the mouse models were localized we decided to use a specific mfERG (cone) pattern for testing the heterozygous *CRB1* mutation carriers. Five out of 7 obligatory carriers demonstrated

specific infero-nasal mosaic retinal dysfunction. The other two carriers showed no abnormalities on mfERG recordings.

The previously mentioned mouse model studies also provided information on Crb1 to be essential in the maintenance of the adherens junctions between photoreceptors and Müller glia cells during light exposure. It was therefore proposed that Crb1 might play a crucial role in the prevention of retinal disorganization and retinal dystrophy. Light may consequently influence the development of retinal disease in the presence of *CRB1* mutations[37]. Difference in light exposure may for that reason also play a role in the mfERG recording differences in the tested *CRB1* mutation carriers.

Interestingly, McKay and co-workers recently reported on a family with autosomal dominant inherited pigmented paravenous chorioretinal atrophy due to a p.V62M mutation in the *CRB1* gene[38]. This is of special interest because the chorioretinal atrophy was subtle in the early disease stage and limited to the inferior quadrant. Disease expression was variable and males where more likely to exhibit a severe phenotype compared to females which remained virtually asymptomatic.

Although the cohort of heterozygous *CRB1* carriers studied is rather small, the majority of our tested individuals display an abnormal phenotype. Moreover, this phenotype is different from previously described heterozygous carrier phenotypes in other tested LCA genes. In the future, mfERG testing of additional heterozygous carriers may specify the ocular carrier phenotype and may also provide characteristic findings in the parents of LCA offspring and add important information on the gene involved in their offspring. MfERG testing in parents of LCA children may therefore be an informative tool in differentiating which gene is involved, potentially facilitating the molecular diagnostic process.

This thesis has supplied information on the usefulness and efficiency of newly developed microarrays that facilitate molecular diagnostics in a portion of autosomal recessive retinopathies. Furthermore, important clinical data on *ABCA4* associated arCRD and arRP as for *AIPL1*, *CRB1* and *GUCY2D* associated LCA and *RPE65* associated early-onset rod-cone dystrophy were provided.

In conclusion, molecular and clinical studies of retinal diseases provide crucial knowledge for the understanding of normal and abnormal retinal development and function.

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7 Summary / samenvatting

Summary

Inherited retinal dystrophies affect approximately 1 in 2000 persons worldwide, which leads to legal blindness in a significant fraction of these patients. Retinal dystrophies display unexpected and surprising clinical and genetical heterogeneity. To date more than 60 genes involved in retinal disease have been identified and at least as many still await discovery. For many years molecular genetic and clinical aspects of retinal dystrophies were studied extensively in order to provide insights in the understanding of these complex disorders and at the same time provide clues for their possible treatment. At least as many questions have been raised as answered, but to date, for the first time in history we are expecting the first human therapy trials for inherited retinal diseases. Most of these putative future therapies are gene specific which emphasizes the importance of knowing the causative gene in individual patients. For the near future this means that all genes involved in inherited retinal diseases should be identified, that rapid, affordable and reliable screening tools become accessible, and that straightforward genotype-phenotype correlations are available.

Autosomal recessive RP displays a wide clinical and genetic variability. To date 19 genes have been cloned and another 5 loci have been mapped. Chapter 2.1 shows that only a minority of arRP patients have disease caused by mutations in *ABCA4*. These patients don't show the typical arRP phenotype associated with mutations in *ABCA4*, being one of an early-onset, severe and highly progressive retinal degeneration. This phenotype however was shown in arCRD patients in chapter 2.2. An association with nystagmus and atrophic nummular maculas in these patients was also provided. Furthermore, two fast and reliable screening methods were used to detect mutations in this gene, i.e. the ABCR500 APEX technology and the arRP Affymetrix Resequencing Array technology. The APEX technique is relatively cheap and detects 60% of the theoretically expected mutations. The resequencing technique is relatively expensive and detects 65% of the mutations. The APEX technique is also of use for mutation analysis in a significant fraction of the arCRD patients, as the majority of the CRD cases carry causative *ABCA4* mutations.

The identification of *ABCA4* mutations is of great importance since studies with *Abcr -/-* mice proved that retinal function could be improved by extensive light reduction and/or treatment with isotretinoin. In chapter 3 we provide genotype-phenotype correlations for a cohort of predominantly Dutch and Belgian LCA patients. As a genotyping tool the APEX LCA microarray was used which proved to be an efficient first-pass screening tool. Mutations were found in ~1/3 of the patients. Mutations were most often found in *CRB1*, followed by *GUCY2D*. Surprisingly, all patients with *CRB1* mutations had either the p.C984Y or the p.K801X mutation. Eight of 12 *GUCY2D* alleles carried the p.R768W mutation, which suggests that this is a founder mutation in the northwest of Europe.

Specific genotype-phenotype correlations were made for three genes (*AIPL1, CRB1, GUCY2D*) that are involved in a substantial fraction of the patients. LCA patients with *AIPL1* mutations showed hyperopia, light perception and essentially normal appearing fundi on early examinations. Fundus abnormalities start to appear in early childhood. After the age of eight years these patients showed a consistent fundoscopic picture, consisting of bull's eye maculopathies with peripheral intraretinal pigment migration. All LCA patients with *CRB1* mutations showed a distinct phenotype with some or all fundoscopic characteristics of the RP12 phenotype. This phenotype consists of relatively normal optic discs and vessels, a distinct maculopathy or macular dysplasia, nummular pigmentations, yellow-white retinal dots, para-arteriolar preservation of the retinal pigment epithelium, Coats-like exudative vasculopathy, vascular sheathing. Most patients showed moderate to high hyperopia, nystagmus and some of them had a keratoconus. Patients with LCA caused by mutations in *GUCY2D* show an extremely severe phenotype of no light perception at initial examination and virtually no signs of improvement later in life. Fundus examinations were relatively normal even later in life.

The *RPE65* gene is generally considered a LCA gene, although most patients described previously with mutations in this gene do not fit the strict LCA criteria. These patients are preferably referred to as suffering from autosomal recessive early-onset retinal dystrophy. In chapter 4, a large Dutch consanguineous pedigree was described. Thirteen out of 14 patients from 10 nuclear families were found to have the same homozygous p.Y368H mutation in *RPE65*. One patient was found to be compound heterozygous for this mutation and the most frequently encountered *RPE65* mutation worldwide, IVS1+5G>A. Clinical analysis showed that all patients suffered from night blindness, nystagmus, extensive central visual impairment with relatively well preserved peripheral vision and an non-detectable electroretinogram (ERG). Remarkably, there was a wide range of visual acuities (VA) in this pedigree and a wide variety in clinical course of vision. The VA deteriorated in approximately one third of the patients, whereas one third showed a stable VA and another third had some improvement of the VA over the years. This clinical variability has been observed in other studies but these differences were attributed to the different functional consequences of different (combinations of) mutations in the *RPE65* gene. Apparently, other genetic and environmental factors play a role in the disease expression pattern of this pedigree.

Since this large Dutch consanguineous pedigree belongs to a rather isolated population, this mutation very likely represents a founder mutation. Mutation analysis in healthy controls from this population reveals that patients with a partner from this population have a risk of 1 in 56 that any of their children will develop the retinal dystrophy.

In previous studies gene-specific ERG abnormalities in obligate heterozygous carriers of *AIPL1* (rod ERG abnormalities)*, GUCY2D* (cone ERG abnormalities)*,*

RPE65 (normal ERGs) and *RPGRIP* (rod and cone ERG dysfunction) mutations, have been reported. In chapter 5, parents of patients with proven *CRB1* mutations underwent clinical ophthalmologic examination including a mfERG to study the possibility of the existence of *CRB1*-specific ERG changes in carriers of *CRB1* sequence changes. Indeed a significant portion of heterozygous carriers of *CRB1* mutations show a specific infero-nasal mosaic retinal dysfunction on mfERG, primarily affecting cone photoreceptors. This specific pattern is probably caused by inferior retinal folds and/or pseudorosettes, which is in accordance with *Crb1* animal models. In the future, ERG testing of parents of LCA infants may aid in the prediction of the gene involved.

Samenvatting

Wereldwijd komen retinale dystrofieën (erfelijke netvliesaandoeningen) voor bij 1 op de 2000 mensen. Een deel van de mensen met deze aandoening is maatschappelijk blind (maximaal gecorrigeerde visus van minder dan 3/60 of een centraal gezichtsveld van 10 graden of minder in het beste oog (International Statistical Classification of Diseases)).

Retinale dystrofieën zijn klinisch en genetisch heterogeen. Momenteel zijn er voor deze aandoeningen ongeveer 60 genen bekend en het is te verwachten dat nog een groot aantal genen in de toekomst ontdekt zal worden. De laatste jaren zijn zowel de moleculair genetische als ook de klinische aspecten van deze dystrofieën bestudeerd om een beter inzicht in deze complexe groep aandoeningen te krijgen. Door dit type onderzoek is er tevens meer kennis omtrent de normale functie van het netvlies vergaard. Daarnaast kunnen deze onderzoeken aanwijzingen opleveren voor eventuele behandelingsmogelijkheden.

Een deel van de toekomstige therapeutische mogelijkheden zal genspecifiek zijn, en daarom is het van belang om het gendefect in individuele patiënten te kennen. Dit betekent dat in de toekomst alle genen betrokken bij erfelijke retinale dystrofieën geïdentificeerd moeten zijn, en dus dat er snelle, goedkope en betrouwbare methoden voor mutatiedetectie toegankelijk moeten zijn. Daarnaast zullen duidelijke genotype-fenotype correlaties een bijdrage leveren aan de identificatie van de oorzakelijke genmutaties in individuele patiënten.

Hoofdstuk 2 laat zien dat patiënten met CRD en mutaties in het *ABCA4* gen een heterogeen klinisch beeld vertonen. Tevens worden nieuwe klinische kenmerken, geassocieerd met dit beeld beschreven, namelijk nystagmus en atrofische maculopathie. Daarnaast worden in dit hoofdstuk twee snelle en betrouwbare *ABCA4* mutatie-screening methodes beschreven, de ABCR500 APEX microarray technologie en de arRP Affymetrix Resequencing Array (AGRA) technologie. Het gebruik van de APEX technologie is relatief goedkoop en detecteert 60% van de theoretisch te verwachten mutaties. In vergelijking met de APEX techniek is het gebruik van de AGRA technologie relatief duur en detecteert deze 65% van de mutaties.

In hoofdstuk 3 worden de genotype-fenotype correlaties voor een cohort van vooral Nederlandse en Belgische LCA patiënten beschreven. Voor de genotypering is de APEX LCA microarray gebruikt hetgeen een efficiënte methode blijkt te zijn, omdat deze 1/3 van de te verwachten ziekte allelen geïdentificeerd heeft. Mutaties zijn het meest frequent in het *CRB1* gen gevonden, gevolgd door mutaties in *GUCY2D*. Alle patiënten met mutaties in *CRB1* blijken drager te zijn van de p.C984Y of de p.K801X mutatie. Acht van de 12 *GUCY2D* allelen dragen de p.R768W mutatie welke zeer waarschijnlijk een founder mutatie in Noordwest Europa is.

In dit hoofdstuk zijn tevens specifieke genotype-fenotype correlaties voor drie genen (*AIPL1, CRB1, GUCY2D*) gemaakt. LCA patiënten met mutaties in *AIPL1* zijn hypermetroop, hebben enkel lichtperceptie en vertonen als baby een vrijwel normale fundus. Vanaf de leeftijd van 6 jaar hebben alle *AIPL1* geassocieerde LCA patiënten fundusafwijkingen in de vorm van een "bull's-eye" maculopathie met beenbalk-achtige intraretinale pigmentveranderingen in de periferie. De LCA patiënten met mutaties in *CRB1* vertonen allemaal kenmerken van het RP12 fenotype. Het RP12 fenotype wordt onder andere gekarakteriseerd door een relatief normale papil met normale vaten, een uitgesproken maculopathie en ronde pigmentaties. Andere klinische kenmerken zijn geelwitte subretinale puntjes, para-arteriolaire sparing van het retinale pigmentepitheel, een Coats-achtige vasculopathie, vaatschedevorming en keratoconus. De meeste patiënten met *CRB1* mutaties uit deze studie zijn daarbij (hoog) hypermetroop en hebben een nystagmus. De patiënten met mutaties in het *GUCY2D* gen vertonen het meest ernstige fenotype. Zij hebben geen lichtperceptie met een vrijwel normaal fundusbeeld, welke niet verandert gedurende hun leven.

In hoofdstuk 4 wordt een Nederlandse stamboom beschreven met daarin een groot aantal consanguine families met 14 patiënten met een autosomaal recessieve, vroeg ontstane retinale dystrofie. Dertien van de 14 patiënten van de 10 kerngezinnen hebben een homozygote mutatie (p.Y368H) in het *RPE65* gen. Eén patiënt blijkt samengesteld heterozygoot te zijn voor deze mutatie en de IVS1+5G>A mutatie, welke wereldwijd de meest voorkomende mutatie in het *RPE65* gen is. Alle patiënten zijn nachtblind en vertonen nystagmus. Verder hebben ze relatief gespaarde perifere gezichtsvelden en een niet te meten electroretinogram (ERG). Binnen deze familie is er een grote verscheidenheid in de gezichtsscherpte en het beloop daarvan. Bij éénderde van de patiënten verslechtert de visus, bij éénderde blijft de visus nagenoeg stabiel en bij éénderde verbetert de visus in de loop der jaren. Deze klinische variabiliteit is in de literatuur al beschreven en wordt toegeschreven aan de verschillende functionele consequenties voor het eiwit door de verschillende *RPE65* mutaties. Het is aannemelijk dat andere genetische- of omgevingsfactoren een rol spelen in de ziekte-expressie van patiënten uit deze familie.

Aangezien deze groep patiënten uit een relatief geïsoleerde populatie afkomstig is, representeert de p.Y368H mutatie waarschijnlijk een founder mutatie. Aanvullende mutatieanalyse van DNA van gezonde personen afkomstig uit deze gemeenschap toont dat patiënten met een niet consanguine partner uit deze populatie een risico hebben van 1 op 56, dat hun kinderen deze retinale dystrofie zullen ontwikkelen.

In hoofdstuk 5 is beschreven hoe ouders van LCA patiënten met mutaties in het *CRB1* gen een uitgebreid oogheelkundig onderzoek ondergaan, inclusief mfERG, zodat mogelijke *CRB1*-specifieke veranderingen in dragers van *CRB1* mutaties bekeken kunnen worden. Eerdere studies tonen genspecifieke ERG's bij obligate heterozygote dragers van mutaties in *AIPL1* (staafjes afwijkingen op ERG), *GUCY2D* (kegel afwijkingen op ERG), *RPE65* (normaal ERG) en *RPGRIP1* (staaf en

kegel afwijkingen). Het blijkt dat een significant deel van de heterozygote dragers van *CRB1* mutaties een specifiek patroon, van voornamelijk kegel dysfunctie in het inferonasale kwadrant, in het mfERG vertoont. In de toekomst kunnen ERG resultaten bij ouders van LCA patiënten, mogelijk bijdragen aan de voorspelling, in welk gen mutaties bij hun kinderen de aandoening veroorzaakt.

Dankwoord

Ik realiseer me dat dit waarschijnlijk de meest gelezen bladzijden uit mijn proefschrift zijn. Er zijn zoveel mensen die een bijdrage hebben geleverd aan het tot stand komen van dit proefschrift. Jullie verdienen het dan ook allemaal om hier bedankt te worden. Een persoonlijk dankwoord voor iedereen zou echter een verdubbeling van het aantal pagina's betekenen. Ik zal me daarom beperken tot een vrij algemeen dankwoord met slechts een aantal persoonlijke dankbetuigingen. Ik hoop niemand teleur te stellen.

Beste Ingeborgh en Frans, om te beginnen wil ik jullie in één adem noemen. Jullie hadden zomaar vertrouwen in dat grietje dat gewoon maar even kwam aanwaaien. Van twee dingen was ik overtuigd, ten eerste wilde ik onderzoek doen, en ten tweede wilde ik oogheelkunde doen. Het is voor mij heel bijzonder geweest dat jullie, alsof het de gewoonste zaak van de wereld was, ineens een onderzoek voor me klaar hadden. Dat ik toen totaal nog niet begreep waar dat over ging, wat het inhield en wat ik moest doen heeft jullie niet weerhouden om met mij in zee te gaan. Het onvoorwaardelijke vertrouwen dat jullie in mij gehad hebben is een grote steun en bron van inspiratie geweest. Ik hoop dat ik jullie niet teleurgesteld heb.

Beste Frans, wat is het geweldig geweest om onder jouw begeleiding dit onderzoek te mogen doen. Tijdens mijn onderzoek ben jij hoogleraar geworden en mag jij gelukkig mijn promotor zijn. Jouw kennis, gedrevenheid, geduld, persoonlijke betrokkenheid en snelle correcties, heb ik als een geweldige steun ervaren. Altijd kon ik op je terugvallen, waar ik ook was en waar ik ook mee zat. Als het nodig was wist je me te motiveren en gelukkig wist je me ook op de juiste momenten af te remmen. Onze voorliefde voor sport heeft ook mooie momenten opgeleverd. Gelukkig houdt onze samenwerking niet op bij het afronden van deze promotie. Jouw familie verdient hier ook een woord van dank, met name de hulp van Diana is onmisbaar geweest (bellen, bellen en nog meer bellen, gezellig lunchen, taart bakken / eten enz).

Beste Ingeborgh, het was ook fantastisch om met jou dit project te mogen doen. Ik heb ontzettend veel respect voor jouw kennis en deskundige benadering voor jouw werk als algemeen oogarts en oftalmogeneticus. Ik heb veel van je mogen leren en leer gelukkig nog steeds veel van je, en dit betreft zeker niet alleen de genetica van retinale dystrofieën. Jouw bijdrage aan alle artikelen in dit proefschrift zijn onmiskenbaar. De heldere, gestructureerde opbouw van de klinische gegevens zijn van jouw hand. We hebben heel wat uren afgebeld waarbij we zo nu en dan soms ook werkelijk over het onderzoek hebben gepraat, in tegenstelling tot wat velen dachten. Bedankt voor alle morele steun en voor de gezelligheid. Hans, ook jij bedankt, jouw kookkunsten worden zeer gewaardeerd.

Beste Robert, jouw naam staat bij 4 van de 5 artikelen. Onze samenwerking is pas later tot stand gekomen, maar zoals blijkt, bijzonder vruchtbaar. Mede dankzij jouw inspanning heb ik 9 geweldige maanden in Montreal gehad. Jouw niet te remmen optimisme voor mijn werk, het werk van anderen, de toekomst voor patiënten met RP en verwante aandoeningen, en het enthousiasme waarmee je al je werk doet en je patiënten benadert zijn voor mij niet alleen zaken geweest waar ik met plezier aan terug denk, maar vormen nog steeds een bron van motivatie en inspiratie.

Ook wil ik al mijn fijne, lieve en gezellige collega's van de afdeling oogheelkunde en vooral van de afdeling Antropogenetica van het Universitair Medisch Centrum Nijmegen bedanken. Ik weet dat ik het geduld van velen regelmatig op de proef heb gesteld. Janneke, Sioe Lie, Karin, Carel, Jeroen, Mariëtte, Han, Hans, Ineke, Lilly, Bellinda, Saskia, Ronald, Hannie, Arjan, Maarten, Bert, Erwin, Mirjam, Sylvia, Ellen, Christel, Iwan, Krysta, Ferry, Stef, Arijit, Pascal, Ersan, Alessandra, Joris, Ilse, Simone, Tuula, Dorien, Jeroen, Helger, Astrid, Imke en natuurlijk Marijke, en vele, vele anderen. Jullie waren er altijd om mijn vragen te beantwoorden, me te hulp te schieten bij mijn labacties (gelukkig kon ik uiteindelijk ook veel alleen!) en het maken van presentaties. Jullie steun bij de hoogte- en dieptepunten van onderzoek, de gezellige pauzes, de goede gesprekken en jullie vriendschap zijn een geweldige herinnering. Het verlaten van het warme Antropogenetica nest is me moeilijk gevallen, en eerlijk gezegd heb ik daar nog steeds vaak moeite mee.

Fijne collega's en oud-collega's uit Het Oogziekenhuis Rotterdam. Hartelijk dank voor jullie steun, belangstelling en gezelligheid. Ook jullie hebben allemaal een aandeel (groot en klein) gehad in de tot stand koming van dit proefschrift. Degene onder jullie die nu denken dat ik eindelijk mijn mond ga houden over "die genetisch dingen" hebben het toch echt mis! Chris, bedankt voor je hulp bij de heisa rondom de promotie.

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Wat was het geweldig om met zoveel verschillende onderzoekers / coauteurs te mogen samenwerken. Ik heb van ieder van jullie veel mogen leren. Het resultaat mag er zijn! Bart, voor jou toch speciaal: "many hugs!".

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Dear Alireza, Alexandra, Irma, Norma, Alison and Joanne, you made my stay in Montreal so wonderful and unforgettable. The memory of my fantastic time in Canada is a sweet memory of you.

Sharola, thank you for making this day even more special!

Mijn paranimfen, Aniek en Anneke. Aniek, we go way back. Net 12 jaar oud en samen op de MAVO. Wat deden we daar? Na de HAVO zijn we ieder onze eigen weg gegaan en toch zijn we er gelukkig steeds voor elkaar geweest. Anneke, jemig, dat wij daar straks samen staan. Jouw hulp in het lab, jouw kennis van de moleculaire genetica van LCA / arRP, maar bovenal jouw vriendschap betekenen veel voor me. Het is gewoon vanzelfsprekend dat wij dit met zijn drieën doen.

Lieve pa en ma, Esther en Emiel. Jullie hebben je vaak afgevraagd waar ik nou toch mee bezig was. Na mijn studie geneeskunde ineens het lab in, en wat deed ik daar? In het weekend de kleinste koffer uitzoeken en dan met de trein (later gelukkig de auto, ook daarvoor dank), naar patiënten en families om vervolgens met diezelfde koffer vol bloedjes terug te komen. Spanning voor praatjes, angst over artikelen schrijven / publiceren, naar Canada……nou het resultaat van wat ik deed ligt nu voor jullie. Hartelijk dank voor jullie geduld, jullie morele steun en het voor me klaar staan. Zonder jullie was dit proefschrift er niet geweest.

Al die fijne mensen die zich hebben laten vangen onder het kopje “collega, coauteur en vriend / vriendin”, sorry dat jullie niet apart genoemd staan. Weet, dat als je niet persoonlijk genoemd wordt, je toch werkelijk niet vergeten bent.

This is it, for now!

Suzanne

Curriculum vitae

Suzanne IJzer werd geboren op 7 augustus 1974 te Groningen. In 1990 behaalde zij het MAVO diploma aan de Aloysius MAVO te Tilburg, waarna in 1992 het HAVO en in 1994 het VWO diploma (Cobbenhagencollege te Tilburg) volgden. Datzelfde jaar startte zij met de studie Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit te Nijmegen, de huidige Radboud Universiteit Nijmegen. Na haar propedeuse behaald te hebben zwaaide zij in 1996 over en begon zij met de studie Geneeskunde aan dezelfde universiteit, welke zij in 1999 afrondde. In 2001 haalde zij vervolgens het arts examen. Gedurende deze periode liep zij tevens stages aan “Universidade de Coimbra”, Portugal en “Universiteit Gent”, België. Vanaf 2001 werkte zij als arts-onderzoeker bij de afdeling Antropogenetica van het Universitair Medisch Centrum St Radboud in Nijmegen. De resultaten van haar onderzoek, dat onder begeleiding van prof. dr. F.P.M. Cremers (afdeling Antropogenetica UMC St Radboud) en dr. L.I. van den Born (Het Oogziekenhuis Rotterdam) plaatsvond, staan beschreven in dit proefschrift. Gedurende de periode april 2004 - december 2004 deed zij een deel van het hier beschreven onderzoek, onder begeleiding van dr. R.K. Koenekoop, aan “McGill University” in Montreal, Canada. Sinds oktober 2005 is zij in opleiding tot oogarts in Het Oogziekenhuis Rotterdam (opleider prof. dr. J.C. van Meurs).
In 2006 kreeg zij de “young investigator award” voor het “XIIth International Symposium on Retinal Degeneration (RD2006)” voor het onderzoek beschreven in hoofdstuk 3. In 2007 mocht zij de eerste Retina Nederland Stimuleringsprijs voor jonge onderzoekers op het gebied van erfelijke oogaandoeningen in ontvangst nemen.

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Color Figures

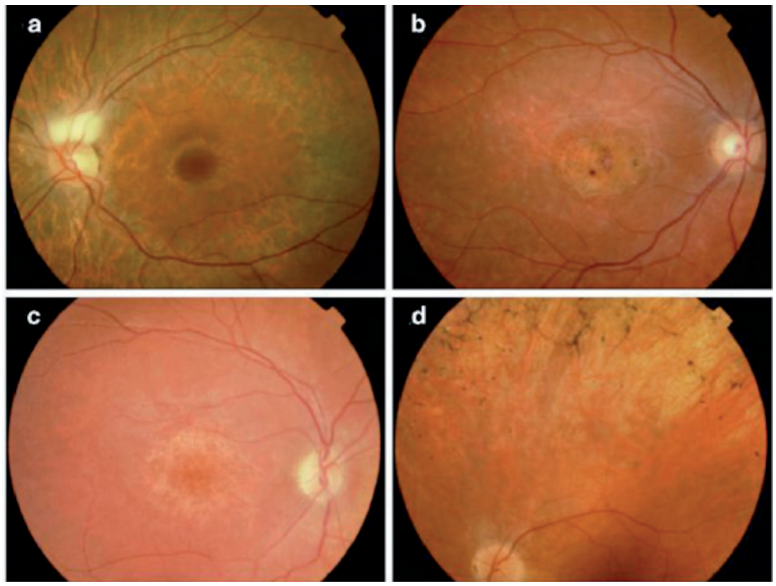

Figure 2.1.1 Fundus pictures of patients with CRD and RP and mutations in *ABCA4*

a. CRD patient 15680 with bull's eye maculopathy and temporal pallor of the optic disc (with myelinated nerve fibers). In the periphery (not visible) there are minor hyperpigmentations but the retinal vessels are of normal size.

b. Chorioretinal atrophy in the posterior pole of CRD patient 16887. Mild granular changes located at mid-peripheral retina (not visible). This patient was initially diagnosed as STGD1, in view of the yellow flecks, which are still faintly visible.

c. CRD patient 16569, taken at age 12, with an obvious pallor of the temporal optic nerve head and atrophic changes in the macula. At that time, the photopic ERG is already nonrecordable and the scotopic ERG is severely decreased.

d. RP patient 17597 shows typical RP features such as bone spiculas in the periphery and attenuated retinal vessels. Large choroidal vessels can be seen in the midperiphery indicative of atrophic changes.

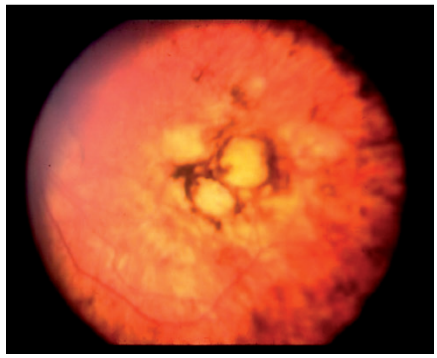

Figure 2.2.2 Fundus photograph of the left eye of patient V:2 with mutations in *ABCA4* (age 46 years)
Note the attenuated vessels, the atrophic lesion in the macula, and bone-spicule pigmentations.

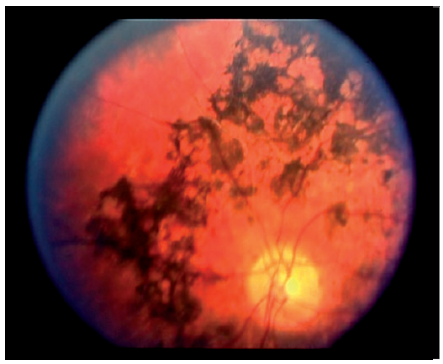

Figure 2.2.3 Fundus photograph of the right eye of patient V:5 with mutations in *ABCA4* (age 43 years)
Note the pale optic disc, moderate attenuation of the vessels and heavy bone-spicule pigmentation in the midperiphery with a relatively spared periphery.

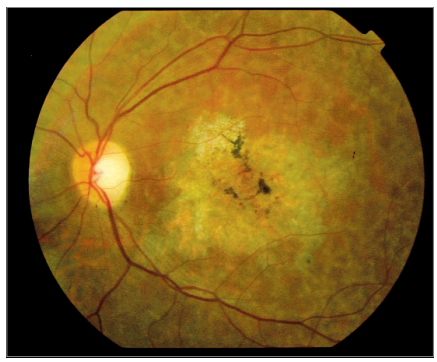

Figure 2.2.4 Fundus photograph of the left eye of patient VI:1 with mutations in *ABCA4* (age 52)
Note the relatively normal optic disc, mild attenuation of the vessels and large atrophic lesion with scattered pigmentations in the macula. The remaining RPE has a lobular atrophic appearance.

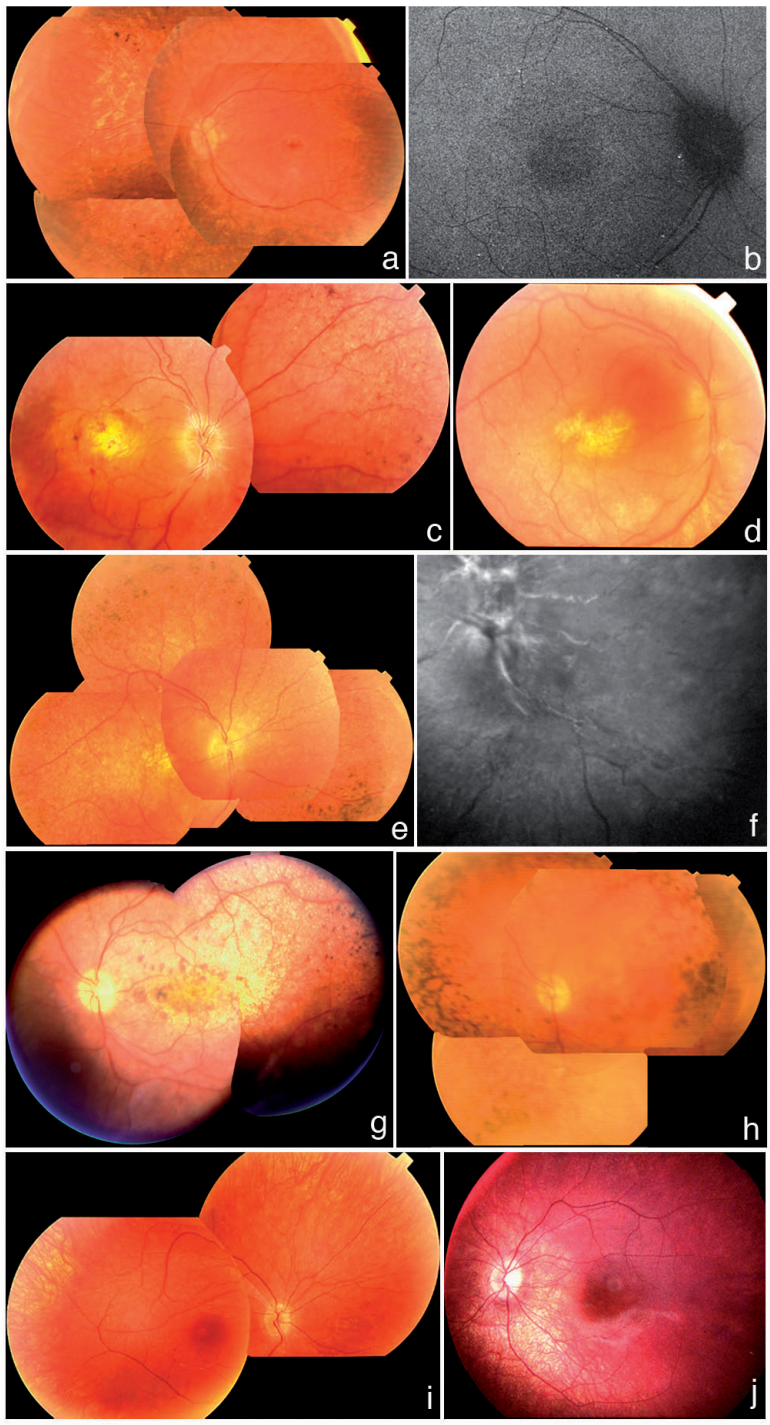
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Figure 3.1 Fundus photographs of patients with LCA

a. Patient 2040V1 (*AIPL1* p.W278X/p.W278W) at age nine: composite of fundus of the left eye (LE), showing macular dystrophy in a bull's eye pattern, diffuse RPE alterations with scarce intraretinal pigment migration with spicular aspect, sub- or deep intraretinal fine white deposits predominantly along vascular arcades.
b. Patient 2040V1 (*AIPL1* p.W278X/p.W278X) at age nine: autofluorescence image of fundus of the right eye (RE), showing fine deposits predominantly along vascular arcades that hyperfluoresce.
c. Patient 21405 (*CRB1* p.C948Y/p.T745M) at age 14: composite of fundus of RE showing an edematous optic disc with perivascular sheating, mild narrowing of the arterioles showing tortuositas with subretinal white dots along arterioles with PPRPE, macular aplasia, atrophic RPE in the periphery with PPRPE, and nummular and a few spicular pigmentations. The posterior pole showed edema with a few intraretinal hemorrhages.
d. Patient 21554 (*CRB1* p.C948Y/c.2842+5G>A): posterior pole of RE at age seven showing an edematous optic disc with preretinal fibrosis, perivascular sheathing, macular dysplasia, and fine, white, deep intra- or subretinal deposits.
e. Patient 21554 (*CRB1* p.C948Y/c.2842+5G>A): composite of fundus of RE at age 12 showing an edematous optic disc with preretinal fibrosis and perivascular fibrotic sheathing without clear PPRPE apart from vessel inferonasal to RE; macular dysplasia remains stable, whereas there is fine nummular intraretinal hyperpigmentation with fine, white, deep intra- or subretinal deposits in the mid- and far periphery.
f. Patient 21554 (*CRB1* p.C948Y/c.2842+5G>A): red free image of the optic disc and inferotemporal peripapillary area of LE at age 12 showing a better visualisation of perivascular fibrotic sheathing.
g. Patient 246V1 (*CRB1* p.K801X/p.Q362X): composite fundus picture of LE at age 14; Note that the optic disc is fairly normal; the macula is dysplastic; limited vascular attenuation with relative PPRPE; nummular hyperpigmentation in the macula and the retinal periphery and multiple white subretinal deposits scattered throughout the fundus.
h. Patient 246V1 (*CRB1* p.K801X/p.Q362X): composite fundus picture of LE at age 30 shows limited visibility due to subcapsular cataract, stable dysplasia of the macula but a dramatic increase in the number of nummular pigmentations in the posterior pole, vascular attenuation; mixed nummular and spicular hyperpigmentation in the retinal periphery and multiple white subretinal deposits scattered throughout the fundus.
i. Patient 21067 (*GUCY2D* p.R768W/p.A946V): Composite of fundus of RE at age 22 showing arteriolar attenuation and mild RPE alterations in the fovea.
j. Patient 23207 (*RPE65* p.R124X/p.F530fs) at age six and a half years: a relatively hypopigmented fundus of LE with mild attenuation of arterioles and diffuse RPE alterations.

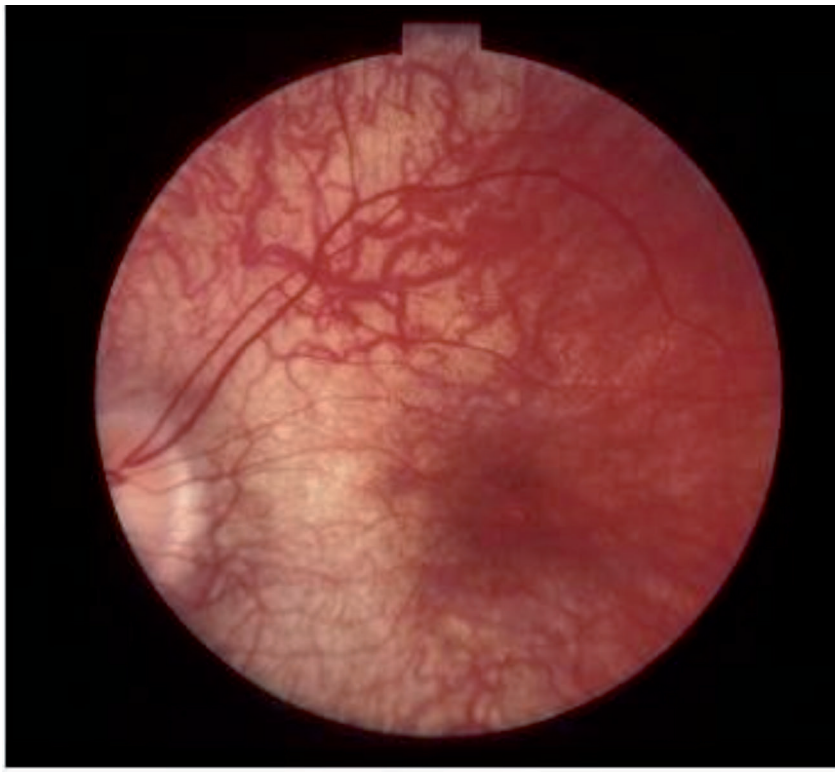

Figure 4.1 Fundus photograph of patient HII-1 at the age of eight years with mutations in the *RPE65* gene
Pale optic disc, moderately attenuated vessels, hypopigmented fundus with a relatively spared maculae

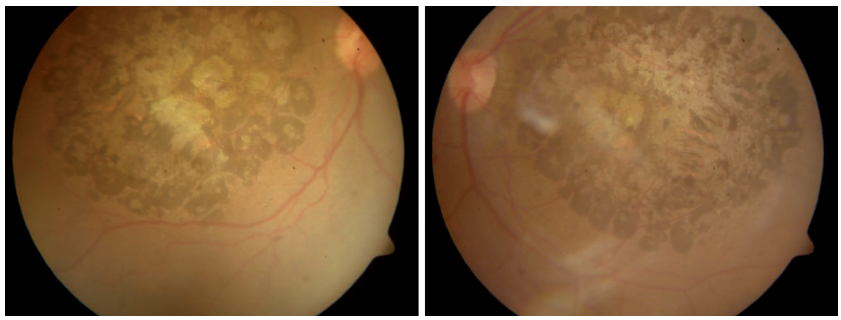

Figure 5.5a + b. Fundus pictures of Family III LCA patient II-1
Shown are the posterior poles of the retinas of the right (5a OD) and left eyes (5b OS) of the affected LCA child with the homozygous p.Q449X mutation in *CRB1* and a heterozygous p. R302L mutation in *AIPL1*. Note the conspicuous maculopathy.

Autosomal recessive retinal dystrophies: genotypes & phenotypes

Suzanne IJzer

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uitnodiging

voor het bijwonen van de openbare verdediging van het proefschrift

Autosomal recessive retinal dystrophies: genotypes & phenotypes

Op maandag 2 juli 2007
om 15.30 uur precies

In de aula van de Radboud Universiteit Nijmegen
Comeniuslaan 2, Nijmegen

U wordt van harte uitgenodigd voor de receptie & borrel na afloop

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